PHYSIOLOGY OF FERTILIZATION OF MAMMALIAN EGGS

by

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This I dedicate to my mother and father, their strength has always been my support - to them my gratitude is boundless.

CONTENTS

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SUMMARY

Cell attached patch recordings have been made from zona-free mouse eggs. Potassium channels which show anomalous and delayed rectification have been observed. The conductance of these channels was as high as 160pS. They have been shown to open and shut independently of one another. Another channel with a conductance of about 50pS has been noted and is believed to be a chloride channel. Calcium channels with mean current amplitudes of 1.10 $\pm$ 0.33pA (mean $\pm$ SD, n=35) and a conductance in the range 7.5 to 20pS, have also been recorded.

Before insemination the membrane potential of zona-free hamster eggs, measured with intracellular electrodes lay in the range -8 to -47mV (whilst bathed in a modified Krebs Ringer solution - called "normal"). In five eggs impaled during this study, the membrane potential was more negative than -61mV. In these eggs calcium action potentials could be evoked by depolarizing pulses. It is suggested that eggs which do not show such action potentials suffer from impalement leaks or have possibly undergone "in vitro deterioration".

Inward channel currents have been observed in synchrony with the rising phase of the action potential.

Preliminary data are presented on whole cell recording experiments.

In zona-free hamster eggs current clamped at potentials more negative than about -80mV, sperm egg fusion was associated with a depolarization, which in some cases elicited an action potential. Such depolarizations or sperm evoked action potentials were also observed in eggs bathed in a solution resembling oviducal fluid, with a potassium concentration of 25mM or in a solution with a sodium concentration of 1mM. Such depolarizations were followed by transient recurring depolarizations.

During fertilization of eggs with low membrane potentials, no response was observed at the time of sperm fusion, but transient recurring hyperpolarizations were later recorded (as seen by other workers).

Similar results were obtained during fertilizations in calcium free solutions containing substituted magnesium and strontium.

"Action currents" have been measured, during fertilization with cell attached patch pipettes. Results of these experiments reveal the presence of (i) spikes superimposed on the repolarizing phase of transient recurring hyperpolarizations, (ii) the latter are sometimes followed by after depolarizations and (iii) these responses were superimposed on a depolarizing shift.

CHAPTER 1 LITERATURE REVIEW

consists of recurring hyperpolarizations

1.22 Fertilization potential in rabbit eggs consists of a slow depolarization, superimposed on which are repetitive diphasic membrane potentials

1.1 Introduction

The investments surrounding the egg constitute major mechanical barriers which the sperm must traverse in order to make contact with the egg. Thus sperm must first interact with the cumulus oophorus cells, then attach and bind to the exterior of the zona pellucida, before penetrating the zona to interact directly with the egg plasma membrane. This is then followed by fusion between the plasma membrane of the penetrating sperm and the egg plasma membrane. Once fusion has occurred, penetration of additional sperm is blocked, in some species, by discharge of the cortical granule contents into the perivitelline space. These act on the zona pellucida, and perhaps also on the egg plasma membrane to block further sperm binding and their subsequent penetration.

In this chapter are discussed the changes that mammalian sperm must undergo, prior to successful in vitro fertilization. After having looked at how the sperm gets to the egg membrane, (section 1.5 the actual fusion process is reviewed. Section 1.10 is a brief summary of the responses of the egg to fertilization, which prevent entry of further sperm and hence prevent polyspermy. Section 1.13 looks at the resting potentials of eggs and 1.14 to 1.22 is a review of the electrical events recorded in various species of eggs at fertilization.

The aim of this study has been twofold:-

a) an attempt to provide further information on the

resting potential of hamster and mouse eggs. Georgiou, Bountra, Bland and House (1983) have provided information showing that hamster eggs do suffer from an impalement leak artifact. A very important question which remains to be answered is "Are mammalian eggs excitable at rest?" (i.e. is their resting potential more negative than the threshold for eliciting action potentials by passing depolarizing current pulses through an impaled intracellular electrode). In this chapter it is mentioned that eggs of echinoderms, echiurans and tunicates are excitable at rest.

b) If the resting potential of hamster eggs is indeed more negative than the threshold for electrically evoked action potentials, then is a sperm penetration (or fusion) in these eggs accompanied by a depolarization. In sections 1.14 - 1.19 results are presented on eggs of echinoderms, echiurans, tunicates and amphibians which show that at fertilization these eggs are accompanied by a depolarization. Whereas in mice and hamster eggs (sections 1.20 and 1.21) no such depolarization is observed during fertilization.

1.2 In vitro fertilization (mammals)

The culture media that have been used for in vitro fertilization range from simple solutions, such as Krebs-Ringer bicarbonate, to complex tissue culture media, such as Medium 199 and Medium F12 (Ham, 1965).

These are usually supplemented with crystalline bovine serum albumin (1 to 10mgml^{-1}). Hoppe and Whitten (1974) have reported that fertilization of mouse eggs in vitro fails to occur when polyvinyl pyrrolidone is substituted for albumin. The function of the albumin is not known. It may possibly stabilize the gamete membranes, chelate the toxic ions, or stimulate the acrosome reaction (see 1.4). Tsunoda and Chang (1975a) have found that the presence of both lactate and glucose in the medium was essential for penetration of a high proportion of rat eggs in their in vitro system. Calcium ions are required for the in vitro fertilization of mouse and rat eggs (Miyamoto & Ishibashi, 1975). The calcium appears to be essential for sperm motility, for the acrosome reaction and for the fusion of the sperm with the egg plasma membrane (Yanagimachi, 1978a) (see 1.8).

Both ovulated eggs and ovarian oocytes have been used. The latter require maturation in vitro but are more readily obtained than tubal eggs from some species, notably man (Lopata, Johnston, Leeton, Muchnicki, McTalbot & Wood, 1974). To obtain an adequate number of tubal eggs superovulation may be induced by gonadotrophins. Yanagimachi and Chang (1964) found no difference in the proportion of eggs penetrated when superovulated as opposed to those eggs which were normally ovulated (golden hamster eggs were used for the in vitro fertilization). Spindle and Goldstein (1975) have shown that the early

developmental capacity of superovulated mouse eggs is similar to that of normally ovulated gametes.

Either ejaculated sperm or more commonly, sperm collected from the cauda epididymis may be used. Rabbit sperm have been shown to develop fertility on reaching the cauda (Orgebin-Crist, 1969), and caudal sperm appear to be as fertile as ejaculated sperm (Overstreet & Bedford, 1974). Hamster sperm may be capacitated by incubation with cumulus cells (Gwatkin, Andersen & Hutchison, 1972). The sperm may be capacitated prior to adding to them to the eggs (as in this study) or alternatively, the capacitating agent and the gametes may be incubated together for a time sufficient for both sperm capacitation and fertilization to take place. The sperm concentration is usually in the range $1x10^6$ to 5×10^6 per ml. Sperm concentrations of $6 \times 10^6 - 6 \times 10^7$ per ml and $2 \times 10^5 - 10^7$ per ml have been reported to be optimal for the in vitro fertilization of hamster (Talbot, Franklin & Fussell, 1974) and mouse (Fraser & Drury, 1975) eggs respectively. But, in these experiments no distinction was made between the requirements for sperm capacitation and those for sperm entry into the eggs. Tsunoda and Chang (1975b) have reported that some mouse eggs are fertilized in vitro even when the sperm concentration is very low (less than 500 per ml). A number of factors can affect the concentration of sperm required, e.g. the adequacy of the medium, the length

of time allowed for interaction of the gametes and the initial proportion of fertile sperm in the sperm suspension (proportion of capacitated sperm, see 1.3). It is possible that only a small proportion of mammalian sperm may actually be fertile, due to errors in spermatogenesis (Cohen & McNaughton, 1974). But the results of Tsunoda and Chang (1975b), showing that in vitro fertilization is possible even with very few sperm, does not support this conclusion.

To prevent evaporation of the sperm suspension medium and possibly also to lower the oxygen concentration to the level pertaining in the oviduct (Mastroianni & Jones, 1964; Ross & Graves, 1974) the medium is usually covered with liquid paraffin. The volume of medium used is usually 40 to 100 μl, although some workers have used volumes up to 5ml (as in this study).

The results of in vitro fertilization may be measured in terms of the proportion of eggs penetrated (e.g. Pickworth & Chang, 1969; Gwatkin et al., 1972), the proportion of eggs with two pronuclei, two polar bodies, and a sperm tail in the vitellus (e.g. Yanagimachi & Chang, 1964; Edwards, Bavister & Steptoe, 1969), or the proportion of eggs undergoing cleavage (e.g. Brackett & Williams, 1968; Whittingham, 1968; Miyamoto & Chang, 1972).

1.3 Sperm Capacitation

Before a mammalian sperm can fertilize an ovum it

must undergo a change in the female genital tract. This phenomenon was first described independently by Chang (1951) and by Austin (1952), who termed the process capacitation.

The first step in capacitation is probably the removal of the epididymal and seminal plasma proteins coating the sperm surface. Evidence for this comes partly from experiments of Johnson and Hunter (1972) who showed that rabbit sperm lose their ability to bind fluorescein - conjugated antibody prepared against seminal plasma, after a 10 hour incubation in the uterus. Oliphant and Brackett (1973a) prepared an antiserum against seminal plasma proteins and showed that this antiserum agglutinated ejaculated sperm, but not those which had been incubated for 12 hours in utero. When ^{14}C labelled antibodies were prepared against rabbit seminal plasma they bound to ejaculated sperm. On incubation of the sperm for six hours in uterine fluid but not in a chemically defined medium, there was a marked decrease in the binding of the antibody.

A second step in the process of capacitation may be an alteration in the glycoproteins of the sperm plasma membrane. Gordon, Dandekar & Bartoszewicz (1975a) found that the lectin, Concanavalin A, binds to the surface of rabbit sperm heads, (and also to the tip of sea urchin sperm - Aketa, 1975). Binding of the lectin to rabbit sperm is probably not due to

epididymal secretions or to seminal plasma components, since it only occurs after the sperm are washed. Furthermore, during capacitation in utero the ability to bind to lectin progressively disappears, beginning at the tip of the head (Gordon, Dandekar & Bartoszewicz, 1974; Gordon et al, 1975a). Treatment of mouse and rabbit sperm with hypertonic salt solutions (a procedure which liberates protein from the sperm, Oliphant & Brackett, 1973b; Singer, 1974), induces the sperm to fertilize eggs in cumulus (Brackett & Oliphant, 1975). Pretreatment of rabbit sperm with hypertonic medium or with glycosidases also induced them to undergo an accelerated acrosome reaction (see 1.4) on transfer to follicular fluid (Oliphant, 1976). These results indicate that peripheral proteins are being removed from the sperm plasma membrane, during treatment with hypertonic medium or glycosidases. Such alteration of the sperm plasma membrane would be expected to prepare it for fusion with the outer acrosomal membrane (see Fig. 1.1), possibly by reducing the net negative charge or by increasing membrane fluidity. This idea is supported by work of Vaidya, Glass, Dandekar and Johnson (1971) who noted a decrease in the mobility of rabbit spermatozoa in an electrophoretic field, following capacitation.

Freeze fracture studies of Koehler and Gaddum-Rosse (1975), have shown that the characteristic longitudinal strands of intramembranous particles present in fractures of the plasma membrane over the

midpiece of guinea pig sperm, undergo dissociation during in vitro incubation in media that promote capacitation. Therefore in future it may be possible to identify the specific plasma membrane alterations responsible for capacitation.

The site of physiological capacitation of sperm within the female tract is uncertain. The initial experiments of Chang (1951 and 1955) established that capacitation of rabbit sperm can take place within the uterus, and this observation has been confirmed for a number of other mammalian species (Bedford, 1970).

The cumulus oophorus is able to induce capacitation of hamster sperm in vitro (Gwatkin et al., 1972). In Medium 199M2 the sperm lose their motility in two hours, but in the presence of the cumulus oophorus rapid motility is maintained for 8 hours or more. The sperm attached to the cumulus cells, remain associated with them for two to three hours and are then released in a capacitated state. During their association with the cumulus cells the sperm are enveloped by the cumulus cell microvilli. Examination under transmission electron microscope shows that fusion between the plasma membranes of the cumulus cells and of the sperm does not take place, but the sperm do become deeply embedded in the cells (Gwatkin, 1977). During this association the cumulus cells appear to alter the plasma membranes of the sperm by secreting glycosidases, since capacitation of golden

hamster sperm by the cumulus oophorus is blocked by glucaro (1-4) lactone, a specific inhibitor of Beta glucuronidase, and by 2-acetamido-2-deoxygluconolactone, a specific inhibitor of Beta-N-acetylglucosaminidase (Gwatkin & Andersen, 1973). Other evidence for a modification in the plasma membrane glycoproteins comes from the work of Gordon et al (1974) who, as mentioned above, observed a progressive loss of Concanavalin A receptors over the anterior tip of the rabbit sperm plasma membrane during capacitation, suggesting the removal or alteration of carbohydrate containing components. Hamster sperm can also be capacitated in vitro by blood serum (Barros & Garavagno, 1970; Yanagimachi, 1970a; Morton & Bavister, 1974; Bavister & Morton, 1974) and by follicular fluid (Yanagimachi, 1969; Gwatkin & Andersen, 1969). But these treatments do not produce sperm capable of penetrating all of the eggs added to them (Mahi & Yanagimachi, 1973).

As a result of capacitation hamster sperm change their pattern of motility from a progressive one, to a bobbing motion of the head, which is believed to be suited to zona penetration (Gwatkin & Andersen, 1969; Yanagimachi, 1970b). Whether this change is mediated by an alteration in the sperm surface or by some other means is not known.

1.4 The acrosome reaction in mammalian sperm

No morphological change has been observed in mouse (Bryan, 1974) or rabbit (Bedford, 1970) sperm lying in

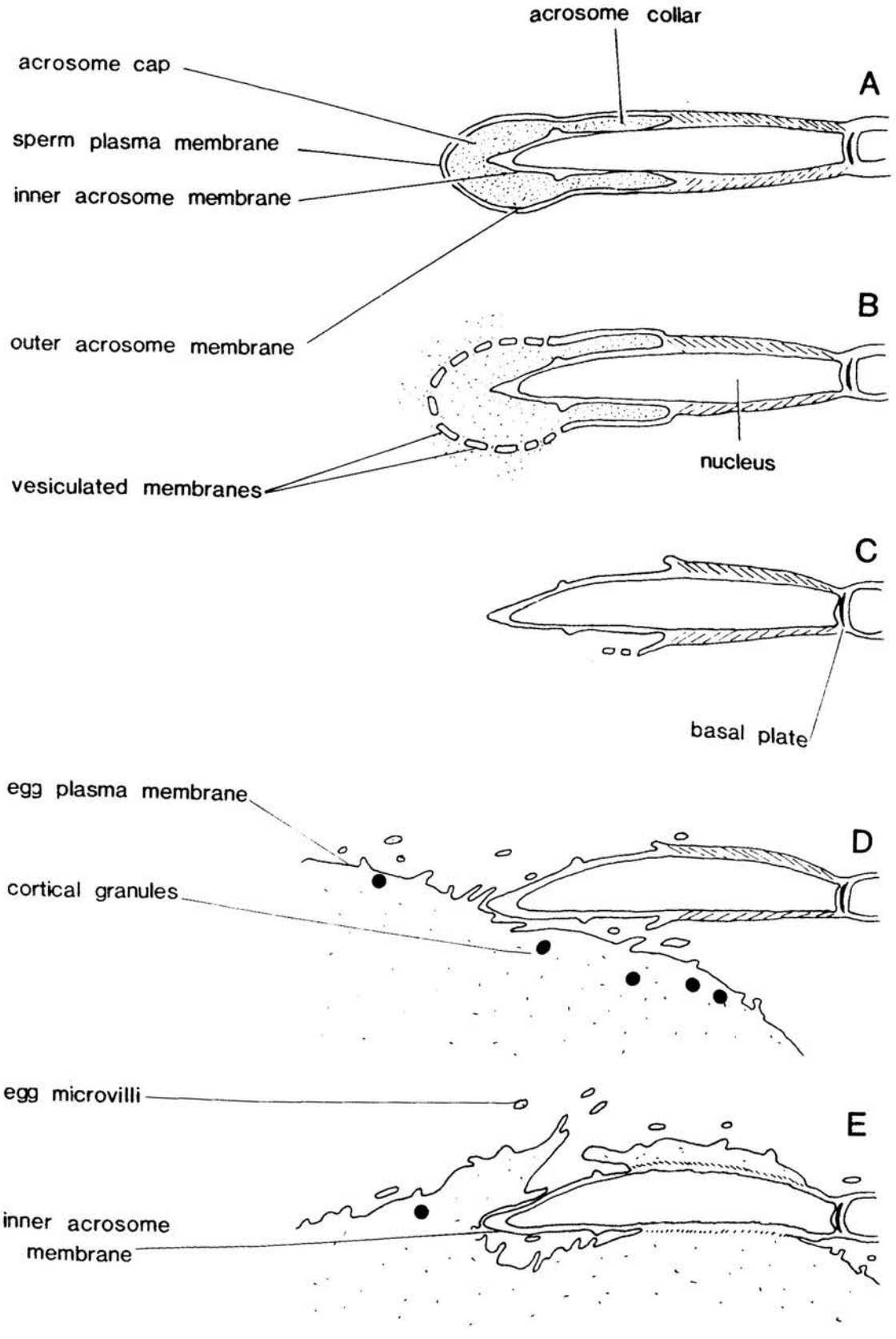
the ampulla of the oviduct. But as the sperm pass through the cumulus oophorus the outer acrosomal membrane may invaginate to form membrane bound vesicles within the acrosome followed by loss of the plasma membrane (Jones, 1973) or multiple fusions may develop between the plasma and outer acrosomal membranes (Franklin, Barros & Fussell, 1970; Yanagimachi & Noda, 1970a). These vesiculation processes indicate the start of the acrosome reaction and probably allow the release of hyaluronidase (Fig. 1.1), which presumably facilitates the passage of sperm through the cumulus. Hyaluronidase release has been used as a criterion for the completion of the in vitro capacitation of hamster sperm (Rogers & Morton, 1973) and for the onset of the acrosome reaction in guinea pig sperm (Rogers & Yanagimachi, 1975a).

The acrosome reaction was originally considered to be part of the process of capacitation (Austin & Bishop, 1958). However Bedford (1970) suggested that the mammalian acrosome reaction can be considered as an event that occurs only in sperm that have been previously capacitated. Since sperm of invertebrates are able to undergo the acrosome reaction immediately, without the need for a period of capacitation, Bedford indicated that it would be "inconsistent to consider the strucural changes of the acrosome reaction as being a step in the process of capacitation".

Vesiculation (1.1B) is accompanied by the loss of

Figure 1.1

Saggital sections of golden hamster sperm (redrawn from Gwatkin, 1977), showing the morphological changes occurring in the head of the sperm, during its travel through the oviduct to its subsequent fusion with the egg plasma membrane. A - in oviduct; B - in cumulus oophorus, the sperm plasma membrane and the outer acrosome membrane have fused in regions to form "vesiculated membranes"; C - in zona pellucida, by now the anterior region of the sperm plasma membrane, the anterior region of the outer acrosome membrane and the acrosome have been lost. Hence the inner acrosome membrane is exposed in preparation for its "contact" with the egg plasma membrane; D - sperm becoming trapped by the egg microvilli; E - fusion occurring between the post-nuclear cap region (also called the post acrosomal region - this is the region of the sperm head immediately to the left of the "basal plate" in A to F) of the sperm plasma membrane and the egg plasma membrane.


acrosome collar
acrosome cap
A
sperm plasma membrane
inner acrosome membrane
outer acrosome membrane
B
nucleus
vesiculated membranes
C
basal plate
egg plasma membrane
D
cortical granules
egg microvilli
E
inner acrosome membrane

the acrosome cap (1.1C). Once the acrosome cap is lost, the sperm rapidly become infertile. An acrosome reaction at the zona surface is probably required to ensure that acrosin (an enzyme believed to be bound to the inner acrosomal membrane, and responsible for forming a tunnel through the zona - see below) is not prematurely inactivated by the tubal environment. Loss of the acrosomal cap observed in the outer regions of the cumulus oophorus (Yanagimachi & Noda, 1970a), may be the result of degenerative changes in supernumerary, i.e. non fertilizing sperm (Zamboni, 1971).

The acrosome reaction in golden hamster sperm, appears to leave a small region of the equatorial segment of the acrosome, intact. Yanagimachi & Noda (1970a) have claimed that this region contains the zona lysin (the term used to refer to the agent responsible for lysis of the zona - called acrosin above), which undergoes vesiculation only during the passage of the sperm through the zona pellucida. But this does not appear to be a general phenomenon, since in the rabbit this region remains unvesiculated even after the sperm has traversed the zona (Bedford, 1968) and in mouse it appears to be lost entirely before zona penetration begins (Thompson, Moore-South & Zamboni, 1974).

When hamster epididymal sperm are suspended in the sperm capacitation medium, a distinct series of events can generally be observed. When the sperm are first "introduced" into the medium, they show an initial burst of activity and soon agglutinate head to head.

After agglutination, sperm motility is markedly reduced for one to one and a half hours, but after this period the motility steadily increases until sperm begin to break away from the agglutinations. At about this time, the motility of the sperm is characterized by vigorous, whiplash movements of the tail, the sperm head tracing figures of eight. This characteristic increase in motility has been called "activation" (Yanagimachi, 1970b), soon after which many sperm undergo the acrosome reaction. When sperm showed both activation and the acrosome reaction they have probably completed the capacitation process, hence these parameters may be visible indications that hamster sperm have attained the capacitated state. Mahi and Yanagimachi (1973) studied the effect of osmolality, hydrogen ion concentration and temperature on activation and acrosome reaction of hamster sperm in vitro. In their study they investigated the effect of incubation temperatures of 42°C to 16°C. Their results indicated that sperm activation, acrosome reaction and survival were impaired at 42°C. But below 42°C, the time required for activation was inversely proportional to the incubation temperature. Osmotic pressures ranging between 193 and 470 mosmol were tested with best rates of sperm activation, acrosome reaction and survival occurring at 230-343 mosmol. The effects of pH values of 5.4-8.6 were tested. Activation, acrosome reaction and survival were best within the range of

7.2-7.8.

It is somewhat surprising that glucose which is present in most in vitro fertilization media, has been found to retard the initiation of the acrosome reaction in guinea pig sperm (Rogers & Yanagimachi, 1975b). But the presence of pyruvate and lactate appears to be required. Toyoda and Chang (1974) have observed that the omission of lactate significantly reduces the population of eggs penetrated. Miyamoto and Chang (1973b) found that the addition of pyruvate to the culture media increased the proportion of motile mouse sperm lacking acrosomal caps and increased the proportion of eggs penetrated.

Yanagimachi and Usui (1974) showed that the addition of calcium (0.2mM) but not magnesium, after guinea pig sperm were incubated for several hours in a calcium free medium causes an acrosome reaction to occur within 10 minutes. This is analagous to the calcium readmission response observed by Douglas and Rubin (1961). They show that in cat's adrenal glands perfused with calcium free Locke's solution, the secretory response of the adrenal medulla to acetylcholine was virtually abolished. But when 2.2mM calcium was introduced back into the perfusate (2.2mM is the concentration of calcium in phosphate buffered Locke's solution) after a 16 min period of perfusion with calcium free Locke's solution, then a vigorous discharge of catecholamines was observed (Douglas & Rubin, 1961). Douglas and Rubin (1961) postulated that

the process of adrenal medullary secretion involves a complex chain of events (referred to as "vesiculation and membrane flow") during which the secretory granules are first attached to the membrane and their contents then extruded through it.

Calcium has also been shown to be essential for the occurrence of the acrosome reaction in several marine invertebrates. Collins (1976) found that 1mM ammonia or a 5 μM solution of the ionophore A23187, which transports calcium across biomembranes, causes sea urchin sperm to undergo an acrosome reaction. Neither treatment is effective when calcium is omitted from the bathing medium. The calcium dependence of the acrosome reaction is probably related to the calcium requirement for fusion. Calcium has been shown to play a central role in controlling many examples of membrane fusion in somatic cells (Poste & Allison, 1973). The triggering of the acrosome reaction by calcium is reminiscent of reports in which provision of calcium has been shown to induce membrane fusion in

a) germ cells (Steinhardt & Epel, 1974; Vacquier, 1975a),

b) diverse somatic cells (Rubin, 1970; Poste & Allison, 1973; Williams & Chandler, 1975) and

c) in model membranes of defined composition (Papahadjopoulos & Poste, 1975; Papahadjopoulos, Poste, Schaeffer & Vail, 1974; Papahadjopoulos, Vail, Jacobson & Poste, 1975).

Since calcium is known to stimulate contractile processes and motility in somatic cells an enhanced rate of uptake of calcium into the sperm may also account for the marked change in the type of motility which is associated with capacitation and the onset of the acrosome reaction.

1.5 Penetration of the zona pellucida by the sperm

Evidence that acrosin is responsible for digesting a pathway for the sperm through the zona pellucida comes first from the fact that acrosin isolated from rabbit sperm will dissolve the zona pellucida of rabbit eggs (Stambaugh & Buckley, 1969). The second line of evidence is that trypsin-acrosin inhibitors inhibit fertilization both in vitro and in vivo (Stambaugh, Brackett & Mastroianni, 1969). The in vivo fertilization of rabbit eggs is also inhibited by incubating capacitated sperm prior to insemination with pancreatic trypsin inhibitor or partially purified rabbit seminal plasma trypsin inhibitor (Zaneveld, Robertson, Kessler & Williams, 1971). Similarly the fertilization of frog eggs is inhibited when sperm are incubated in naturally occurring or synthetic trypsin inhibitors prior to insemination, (Greenslade, McCormack, Hirsch & Danvanzo, 1973). Although these studies tend to implicate acrosin as the "zona lysin" (agent responsible for lysis of the zona), it should be noted that relatively high concentrations of the inhibitors were required for fertilization inhibition.

Hence this raises doubts about the specificity of action of these inhibitors. Miyamoto and Chang (1973a) found that they were unable to inhibit the fertilization of hamster eggs in vitro with soya bean trypsin inhibitor. TLCK (synthetic trypsin inhibitor) was inhibitory at $100 \mu g ml^{-1}$, but at this concentration it impaired sperm motility. Another reservation arises from the fact that in none of these studies has it been shown that the acrosome reaction was able to take place in the presence of the inhibitors. The observed failure of inhibitor treated sperm to penetrate and fertilize eggs could be due to an inhibition of the acrosome reaction rather than a direct inhibition of the enzyme itself.

If one assumes that acrosin is the lytic enzyme which allows sperm to tunnel through the zona then the question of its location is raised. Bedford (1968) has proposed that acrosin is bound to the entire inner acrosomal membrane. Yanagimachi and Noda (1970a) have suggested that it is located in the electron dense equatorial segment of the acrosome, which in hamster sperm vesiculate, during the tunnelling process. Evidence for the equatorial localisation was put forward by Barros, Fujimoto & Yanagimachi (1973) who observed that a prolonged incubation of hamster sperm in serum, which exposed the entire inner acrosomal membrane, resulted in a loss of fertility. But these observations do not prove that acrosin is located only

in the posterior portion of the acrosome, since the medium which they employed may have inactivated the exposed enzyme. Furthermore the persistence of the posterior plasma and outer acrosomal membranes is not a general phenomenon. In the mouse they normally disapppear completely before penetration begins (Thompson et al., 1974). It seems likely therefore that the zona lysin is a peripheral protein located over most if not all of the inner acrosomal membrane.

Scanning electron microscopy on hamster gametes in vitro has shown that the sperm plough a deep furrow in the zona surface. The sperm then traverses the zona pellucida obliquely, although occasionally penetration can occur radially (Yang, Lin, Wang & Chang, 1972). Movement through the zona appears to be facilitated by the bobbing movement of the head which develops following capacitation and presumably brings the acrosin on the inner acrosomal membrane into close association with zona material. In rodents the process may also be aided by organization of the perinuclear material into a relatively rigid structure, the perforatorium (Yanagimachi & Noda, 1970a).

Green and Purves, (1984) have suggested that the sperm penetrate the zona solely by mechanical means, without the assistance of an enzyme. The thickness of the perforatorium varies from about 20-25nm in the rabbit, to about 80nm in the guinea pig sperm. The cross sectional area presented by a penetrating sperm before the acrosome reaction would be typically in the

order of $2\mu m^2$. Measurements for rabbit sperm taken from Bedford (1968), give a maximum thickness for the head of 400-450nm. The width is about 5μm. After the acrosome reaction, the cross-sectional area presented by the perforatorium is about $0.1\mu m^2$. Therefore on the mechanical theory of penetration, the perforatorium acts as a sharpened leading edge, to concentrate stress induced in the zona. Calculations by Green and Purves (1984), on the force exerted by sperm, indicate that if the sperm penetrates the zona mechanically, then the zona must behave as a liquid. Experimental evidence on the structure of the zona is consistent with this view. This theory offers no explanation for the species specificity of zona penetration, discussed below.

The time required for the sperm to traverse the zona pellucida appears to be variable. Yang et al (1972) removed cumuli oophori containing eggs and sperm from the hamster oviduct and observed that 4-22 minutes was required for zona penetration. After traversing the perivitelline space (a rapid process requiring only 1-2 seconds: Yanagimachi, 1966), the sperm moved some distance away from its point of entry, before fusing with the vitelline membrane (egg plasma membrane).

Penetration of the zona pellucida is species specific, e.g. capacitated hamster sperm do not enter the zona intact rat egg (Barros, 1968). Although a limited binding may occur to the zona pellucida by sperm of a heterologous species (Hartmann, Gwatkin &

Hutchison, 1972). It has also been observed that capacitated guinea pig sperm do not penetrate zona intact hamster eggs, although they will fuse with zona free eggs (Yanagimachi, 1972b) (see section 1.7).

1.6 Fusion of sperm with the egg membrane

Studies with hamster gametes in vitro have shown that the microvilli of the vitellus wrap around the head of the sperm and fuse with its plasma membrane in the post-acrosomal region (Yanagimachi & Noda, 1972). It is not known why the inner acrosomal membrane of mammalian sperm cannot fuse with the egg plasma membrane, but it could be due in part to a lack of "fluidity" of the inner acrosomal membrane. In invertebrates the inner acrosomal membrane at the time of the acrosomal reaction, stretches enormously forming the apical process (or acrosomal process) (Colwin & Colwin, 1967; Dan, 1967). The membrane of the acrosomal process may result from either the stretching of the intrinsic components of the inner acrosomal membrane and/or from de novo synthesis from a precursor substance. In any case, the membrane of the acrosomal process of invertebrate sperm must be rather fluid in nature. In mammals, the inner acrosomal membrane remains "unchanged" during and after the acrosomal reaction. Using ferritin conjugates of wheat germ lectin, Yanagimachi (1978b) labelled acrosome reacted mammalian sperm. Some of these he kept at $4^{o}C$ and the remainder he incubated at $37^{o}C$. It was found that the

inner acrosomal membrane was evenly labelled by the conjugates in sperm incubated at both temperatures, suggesting that the components of this membrane almost totally lack mobility.

The primary function of the inner acrosomal membrane of mammalian sperm appears to be the recognition and the penetration of the zona pellucida (Yanagimachi, 1977), not the fusion of the sperm with the egg. Although possibilities exist that (1) the inner acrosomal membrane assist the sperm in firmly attaching to the egg surfaces and (2) enzymes localized in the inner acrosomal membrane renders the egg plasma membrane capable of fusing with spermatozoa (Morton, 1975). At the time of normal fertilization, a sperm which has passed through the zona pellucida and is about to fuse or is fusing with the egg has already completed the acrosome reaction (Yanagimachi & Noda, 1970a). Results of Yanagimachi and Noda (1970b) showed that both acrosome intact and acrosome reacted hamster sperm are able to stick to the egg surfaces, but only the acrosome reacted sperm fused with the eggs. Acrosome intact sperm may become trapped by the egg microvilli but they never fuse with the eggs. This has also been found to be true of the guinea pig (Yanagimachi, 1972a). It appears that the sperm plasma membrane covering the post-acrosomal region of the sperm head undergoes some physiological changes concomitant with, or as a result of the acrosome

reaction, and these changes in the membrane make the sperm capable of fusing with the egg plasma membrane. The nature of these changes is unknown at the present time, but they may be due to an alteration or removal of sperm surface components that hinder a close approximation of the sperm membrane with the egg plasma membrane.

In some mammals (e.g. the Chinese hamster and field vole) the tails of the sperm often detach from the heads during fusion with eggs and are not incorporated into the egg cytoplasm (Austin, 1961). In most mammals, however, the entire length of the sperm tail is incorporated into the egg by fusion between the plasma membranes of the sperm tail and egg. This fusion proceeds from the proximal to the distal end of the tail. In some cases straight-forward fusion occurs between the egg plasma membrane and the sperm tail membrane. In some other cases, however, the sperm tail is encapsulated by egg microvilli before fusion takes place. After incorporation into the egg cytoplasm, the middle piece, mitochondria and the axial filament of the sperm tail eventually disintegrate (Szollosi & Hunter, 1973). It is unlikely that these sperm elements make any significant contribution in embryonic development.

1.7 Species specificity of fertilization in mammals

The main mechanisms by which hybridization in mammals is prevented are the "physiological" and

"behavioural" separation of males and females of different species. The physiological incompatibility of sperm, in the female genital tract of another species, as well as the inability of hybrid embryos to develop may also play a part in preventing hybridization. Nevertheless, the eggs and sperm of each species have distinct species specific properties and are capable of fusing with the opposite gametes only of the homologous species. The most prominent site for the species specificity in mammalian fertilization is the egg zona pellucida (Yanagimachi, 1977). Sperm of the foreign species are generally unable to attach (bind) to the zona, and even if they attach they cannot penetrate it. The lack of a strong species specificity at the level of the egg plasma membrane is evident from experiments in which zona free eggs were inseminated with sperm of heterologous species. Zona free hamster eggs, are penetrable by sperm of the rat, mouse (Hanada & Chang, 1972 & 1976), guinea pig (Yanagimachi, 1972b; Barros, Berrios & Herrera, 1973) and even human (Yanagimachi, Yanagimachi & Rogers, 1976). At least in the combination of hamster eggs with guinea pig or human sperm the sperm penetration was due to a true membrane fusion between gametes, not to a phagocytic engulfment of sperm by the egg. It is interesting to note that the successful fusion of sperm with heterologous eggs requires the sperm acrosome reaction (Yanagimachi, 1972b; Barros et

al., 1973; Yanagimachi et al., 1976) just as in the case of normal fertilization.

Visible indications of egg activation (the fusion of cortical granules with the egg membrane and the resumption of meiotic division) following the fusion of heterologous sperm are quite "normal" (Yanagimachi, 1972b; Yanagimachi et al., 1976) indicating that the mechanisms by which the sperm triggers egg activation are not strictly species specific. The development of egg and sperm pronuclei and their union also appear to proceed "normally" indicating that the mechanisms controlling pronuclei development and union are again not strictly species specific. The incompatibility of the sperm nucleus (chromosomes) with the egg cytoplasm of the unrelated species must be exhibited in later stages of development.

1.8 Calcium requirement for sperm egg fusion in mammals

Experiments by Yanagimachi (1978a) showed that hamster, guinea pig and human sperm moved much less actively in calcium free media (Ca free) and in media free of calcium and magnesium (Ca + Mg free media). These experiments provided further evidence for the important role of extracellular calcium in regulating the movement of sperm (Morita & Chang, 1970; Miyamoto & Ishibashi, 1975). The failure of sperm egg fusion in Ca free and Ca+Mg free media was not due to the poor

motility of the sperm. Since actively motile hamster and guinea pig sperm were seen to attach to hamster and guinea pig surfaces. Sperm that attached to egg surfaces in Ca+Mg free media were observed to penetrate the eggs upon transfer to calcium containing media. This indicates that the failure of sperm penetration in Ca+Mg free medium was due to a specific inhibition of the sperm egg fusion process rather than an irreversible damage to the eggs and/or sperm caused by the absence of calcium in the bathing medium (Yanagimachi, 1978a).

Magnesium, strontium, barium and manganese could replace calcium in triggering sperm fusion (Yanagimachi, 1978a). Similarly in sea urchins acrosome reacted sperm are unable to fertilize eggs in an artificial sea water lacking both calcium and magnesium (Sano & Mohri, 1977). In sea water containing 48-50mM magnesium, however, these sperm can effect fertilization regardless of the presence or absence of calcium (Sano & Mohri, 1977). This may be interpreted as indicating that high concentrations of magnesium can replace calcium in the processes of sperm egg contact and fusion. During homologous fertilization of hamster eggs, calcium was found to be the most potent inducer of sperm egg fusion - 100% penetration with a calcium concentration of 0.2mM. Higher concentrations of barium, strontium and magnesium were required for 100% penetration rates

(about 2-5mM). These results are those obtained by Yanagimachi (1978a). Manganese appeared to be as potent as calcium, but was harmful to the eggs, causing extensive cytolysis.

1.9 Cortical granules in mammalian eggs

The cortical granules in the mammalian eggs were first described by Austin (1961) in the eggs of golden hamster. Their diameters were given as being in the range 0.1-0.5 μm and they occur near to the egg membrane. The cortical granules in the unfertilized eggs of hamster, appear to undergo spontaneous release of their contents into the perivitelline space. This may lead to the loss of fertilizability in these eggs (Yanagimachi & Chang, 1961). The results obtained by Longo (1974a) differ from those reported by Yanagimachi and Chang (1961), for even though there is a loss of cortical granules from the spontaneously activated egg, it is not complete, i.e. only a portion of the cortical granules from the activated egg is released whilst a considerable number are retained. In the spontaneously activated egg, the decrease in the number of cortical granules occurs as a budding of the surface of the egg, into the perivitelline space. A similar loss of cortical granules has also been reported in the aged egg of the mouse (Merchant & Chang, 1971) and the rabbit (Longo, 1974b). According to Longo (1974a,b) this loss of cortical granules during aging does not simulate cortical granule

dehiscence or breakdown during insemination and thus has been considered a part of the degenerative processes of the egg.

1.10 The egg's block to polyspermy

The eggs of most mammals at ovulation are metabolically relatively inert cells arrested in metaphase II of meiosis. In response to an activation stimulus (see below) normally provided by fertilizing sperm, but induced artificially by a number of parthenogenetic agents, the egg resumes meiosis, undergoes a cortical reaction and becomes more active metabolically. In the cortical reaction (see below) the egg undergoes the exocytotic release of its cortical granules, a process that results in the formation of a new mosaic plasma membrane from the fusion of limiting cortical granule membranes with the egg plasma membrane. At the same time the cortical granule contents released into the perivitelline space come into contact with the egg plasma membrane and the zona pellucida.

The formation of an effective block to multiple sperm penetration is crucial for normal development, because entry of more than one sperm leads to abnormal embryogenesis and early death. The cumulative effect of several factors, limits this outcome in mammals by:-

i) the restricted number of sperm reaching the site of fertilization

ii) the protective effect of the egg's associated cumulus cells and

iii) the egg's block to polyspermy responses. These include the egg plasma membrane block to polyspermy (vitelline reaction) and the zona reaction. Each of these is discussed below.

Austin and Bishop (1957) recovered eggs from naturally mated animals and quantitated the incidence of multiple sperm penetration of the vitellus and of the zona pellucida. On examination of eggs from several species, it was concluded that blocks to polyspermy occur to varying degrees at two levels, i.e. the zona pellucida and the egg plasma membrane. Thus in one group of animals (hamster, dog, sheep) sperm were never seen in the perivitelline space, supporting the existence of a primary block at the zona level. In a second group (rabbit, pocket gopher and probably mole) multiple sperm were recovered in the perivitelline space of monospermic embryos indicating a block at the egg plasma membrane. A third group is intermediate between the two groups mentioned above, in which occasionally sperm are observed in the perivitelline space (mouse, rat, guinea pig, ferret and cat).

a) Cortical Reaction

Fusion of the cortical granules with the egg plasma membrane and a discharge of their contents into the perivitelline space occurs when the sperm reaches

the vitellus (Szollosi, 1967). Work on hamster gametes in vitro has shown that the trigger for this so called cortical reaction is the fusion between the egg plasma membrane and the sperm plasma membrane. Simple contact between these two membranes is not sufficient to initiate the reaction (Gwatkin, Rasmusson & Williams, 1976). Penetration of the sperm into the vitellus is not required since it was observed that capacitated sperm could initiate the reaction even after they had been frozen and thawed, so as to prevent penetration. The cortical reaction is propagated around the egg starting at the point of association of the fertilizing sperm with the egg plasma membrane (Braden, Austin & David, 1954).

Gwatkin et al (1976) have shown that various treatments are able to induce the cortical reaction. These include (a) application of positively charged particles to the vitelline surface and (b) exposure of hamster eggs to neuraminidase, to lectins (Concanavalin A and phytohaemagglutinin), to the monovalent ionophore boromycin and to 1,3-bis (4-chlorocinnamylideneamino) guanidine. These workers have suggested that all these agents may act by inducing a depolarization of the vitelline membrane, but no experimental evidence of this has been reported.

Studies by Steinhardt, Epel, Carroll & Yanagimachi (1974) have shown that the cortical reaction can also be induced in the sea urchin, toad and hamster eggs with the ionophore A23187. For hamster gametes a 2

minute exposure to 3μM solution is sufficient to block fertilization. A23187 is a transporter of divalent cations such as calcium, across plasma membranes (Pressman, 1973; Schaeffer, Safer, Scarpa & Williamson. 1974). Since A23187 induced the cortical reaction in both the sea urchin and hamster eggs in the absence of calcium or magnesium in the bathing media, Steinhardt et al. (1974) have suggested that the ionophore may be releasing a divalent cation from one or more of the intracellular stores (rather than transporting divalent cations into the egg from the bathing medium). This is probably calcium since the bulk of magnesium in unfertilized sea urchin eggs is in the free form, whereas the bulk of the calcium is in the bound form (Steinhardt & Epel, 1974). An increase in dialyzable (i.e. free) calcium has been recorded following the fertilization of sea urchin eggs (Nakamura & Yasumasu, 1974). Since the binding of calcium to one side of a phosphatidylserine bilayer has been observed to alter its polarity (Wobschall & Ohki, 1973), A23187 may also be inducing the cortical reaction by causing a depolarization of the hamster egg membrane. It is possible that the calcium release may affect the cortical granules directly.

Vacquier (1975a) has obtained sea urchin oolemma preparations with attached cortical granules, by sticking the eggs on to a layer of protamine and then shearing off the unattached membranes and egg

cytoplasm. When calcium was added to such preparations the granules discharged in a propagated way. This preparation (called the "isolated cortex") has been used to study the mechanism of exocytosis because (a) the cytoplasmic face of the egg membrane with it's associated cortical granules is accessible to experimental manipulations and (b) the cortical granules are sufficiently large, so that granule discharge may be observed by light microscopy (Whitaker and Baker, 1983).

It has been shown that in order for phospholipid vesicles to fuse with bilayers they must first increase in size, i.e. "swell" (Akabas, Cohen & Finkelstein, 1984). Recent studies on sea urchin eggs has indicated that the cortical reaction is prevented when the osmolarity of the medium surrounding the eggs is increased from one to two osmol kg^{-1} (with sucrose or sodium sulphate or stachyose) (Zimmerberg & Whi taker, 1985). In these experiments, attempts were made to initiate the cortical reaction by fertilization or activation with the calcium ionophore A23187. The inhibition is reversible because if the eggs are returned to sea water after ionophore treatment in the presence of a high osmolarity solution, the egg cortical granules undergo a complete exocytosis. The cortical granules shrink in solutions of high osmolarity, suggesting that if swelling of the granule is prevented then the cortical reaction is inhibited. Due to the incorporation of the limiting membranes of

the cortical granules into the plasma membrane of the egg during the cortical reaction, there is an accompanying two to threefold increase in the membrane capacitance (Jaffe, Hagiwara & Kado, 1978; Cole & Spencer, 1938). Such an increase in capacitance is inhibited when the eggs are bathed in stachyose and subsequently treated with ionophore (although the calcium associated membrane depolarization caused by the calcium ionophore is observed). By employing the above mentioned isolated cortex preparation, it has been shown that the granules shrink when exposed to high osmolarity solutions of stachyose and swell slightly when subsequently exposed to calcium (Zimmerberg & Whitaker, 1985). The granules "normally" undergo exocytosis in response to micromolar concentrations of calcium (Baker & Whitaker, 1978; Whitaker & Baker, 1983; Sardet, 1984). The scheme postulated by Zimmerberg and Whitaker (1985) is that calcium in normal conditions induces a swelling of the granules which is sufficient to lead to fusion. But in high osmolarity solutions, the calcium induced swelling is insufficient to cause fusion (the calcium induced swelling does not exceed the shrinkage due to the high osmolarity solution). Calcium may also lead to a closer apposition of the cortical granules with the inner surface of the egg plasma membrane. The calcium induced swelling of the cortical granules may be accomplished in a number of ways. Calcium could alter

the ion permeability, by opening calcium activated channels, resulting in an influx of ions into the granule with water following. Alternatively calcium could modulate pump activity or activate osmotically inactive granular constituents. On the other hand, the cortical granules could be sustaining a hydrostatic pressure. So that when the granules and the plasma membrane come into intimate contact as a result of calcium stimulation, the cortical granules burst and fuse, because the radius of curvature in the region of contact is greater than elsewhere, and thus the vesicles cannot sustain the pre-existing hydrostatic pressure.

b) Vitelline reaction

Due to the cortical reaction, much of the original egg membrane is reconstituted from the membranes of the cortical granules (Graham, 1974). This change is called the vitelline reaction and it functions to prevent sperm entry. It is the primary block to polyspermy in the few mammalian species which lack a zona reaction (see below).

Rabbits fall into this category, although most in vitro studies on the block to polyspermy have involved performing experiments on zona-free mice eggs. It is important to note that insemination of mice eggs with "normal" concentrations (10^5 sperm/ml) of sperm result in very high levels of polyspermy (Wolf, 1978). Hence the response would be better called the block to

multiple or unlimited sperm penetration. In studies on the mouse (Pavlok & McLaren, 1972) and rat (Toyoda & Chang, 1968), evidence for a block came from the observation that the penetration of zona-free eggs was limited and that polyspermy levels were often comparable to those of zona intact controls. But sperm quality and number was largely ignored in these studies and the zona was removed with proteases. The latter treatment reduces the number of sperm fusing with the egg (Wolf, Nicosia & Hamada, 1979). The block response has more recently been studied in mechanically denuded zona-free eggs of the mouse, where reinsemination experiments have estimated that a block time occurs 40 minutes after the initial sperm penetration (Wolf, 1978). The hamster does not show a block at this level, as indicated by the observation that capacitated sperm in this species, are capable of penetrating zona -free eggs at both the pronuclear and the early cleavage stages (Usui & Yanagimachi, 1976). Evidence against a cortical granule role in the plasma membrane block, comes from a study with zona-free mouse eggs, in which premature loss of cortical granules was triggered by ionophore exposure or by mechanical stimulation (Wolf, Nicosia & Hamada, 1979). Egg exposure to ionophore resulted in a 50% loss of the granules, as estimated by TEM (transmission electron microscopy) examinaton of thin sections, while complete granule loss accompanied mechanically induced egg activation. The fertility of these activated eggs was identical to

that of controls as measured by the percentage of eggs penetrated and by the mean number of sperm per egg. Furthermore, ionophore treated eggs subsequently underwent a normal sperm induced block response, leading the authors to conclude that cortical granules are not involved in the plasma membrane block in the mouse.

Resistance of the vitelline membrane to sperm entry appears to develop more slowly than the zona reaction. Barros and Yanagimachi (1972) have estimated that in the hamster egg 2-3.5 hours are needed for the vitelline block to develop compared with less than 15 minutes for the zona reaction, to become effective. Following fertilization the rabbit vitelline membrane binds more Concanavalin A (Gordon, Fraser & Dandekar, 1975b) indicating a change in the distribution of macromolecular components in the vitelline membrane.

c) Zona reaction

The concept that the products of cortical granule discharge alter the zona pellucida so that sperm can no longer penetrate it, was originally proposed by Austin and Braden in 1956. In a few mammalian species, e.g. the rabbit (Braden et al., 1954), this reaction is absent.

Proof that the zona reaction is produced by the cortical granule contents was first provided by Barros and Yanagimachi (1971), who collected material discharged from fertilized hamster vitelli and found

that eggs pretreated by it became infertile. Gwatkin, Williams, Hartmann & Kniazuk (1973) found that cortical granule material collected from fertilized membranes, altered the zonae pellucidae of unfertilized eggs so that sperm did not bind to them, and hence fertilization was prevented.

A trypsin like protease responsible for elevating the vitelline layer and inactivating the receptor for sperm, has also been demonstrated in the cortical granules of sea urchin eggs (Vacquier, Epel & Douglas, 1972a; Vacquier, Tegner & Epel, 1972b; Vacquier, Tegner & Epel, 1973). This enzyme appears to exist in the cortical granules as a zymogen, since it is relatively inactive when liberated in the presence of a chelating agent (Vacquier, 1975a). The addition of calcium but not magnesium causes a tenfold increase in the enzyme activity. Carroll and Epel (1975) have resolved the protease into two components, one which breaks the attachments between the vitelline layer and the plasma membrane and the other which appears to alter the egg surface so that sperm can no longer bind to it. The former they call vitelline delaminase and the latter, sperm receptor hydrolase. Carroll and Epel (1975) have suggested that the simultaneous release of these two proteases may explain the coincidence of membrane elevation and sperm detachment.

In addition to the inactivation of receptor sites for sperm and the elevation of the vitelline layer

which occur following fertilization in the sea urchin, there is a third change. This is a "hardening" of the vitelline layer that renders it insoluble in mercaptoethanol, which dissolves the vitelline layer of unfertilized eggs (Lallier, 1970). This hardening of the vitelline layer is prevented if the eggs are fertilized in the presence of penicillamine and semicarbazide, which appear to act by preventing the formation of cross links, (including disulphide links) between polypeptide chains. This result suggests that the hardening of the vitelline layer may be due to the formation of such new cross links (Lallier, 1971).

A similar hardening of the vitelline layer appears to occur in the zona pellucida of mammals. Some workers have suggested that the increased resistance of zona pellucida to digestion by proteases and mercaptoethanol is involved in the block to multiple sperm entry (Szollosi, 1967). This may be incorrect, since hamster eggs exhibit a strong block to multiple sperm penetration of the zona pellucida, but no change in it's solubility properties, while rabbit eggs in which there is no such block exhibit a marked resistance to solubilization by proteases and mercaptoethanol following fertilization.

This hardening or toughening of the zona pellucida is distinct from the block to multiple sperm entry, which involves the digestion of sperm receptor sites. It may be important in protecting the egg during cleavage as it is transported down the oviduct to the uterus. The

presence of new intermolecular bonds would be expected to increase the resistance of the zona pellucida to protease digestion. Two components of the zona reaction are not necessarily indicative of the same event. For example, zona hardening has been reported to occur (Gould, Zaneveld, Srivastava & Williams, 1971) in rabbit which is a species which does not show a zona block, i.e. in the absence of changes in zona penetrability (Bedford & Cross, 1978). In the hamster on the other hand dramatic changes in zona penetrability occur, at least in eggs recovered from natural matings, in the absence of demonstrable zona hardening (Inoue & Wolf, 1975; Gwatkin, 1977). Also in mice eggs zona hardening is observed within one to two hours after zona penetration, while changes in sperm binding to the zonae are not seen until six to ten hours after fertilization in vivo (Inoue & Wolf, 1975).

1.11 The involvement of calcium in the activation of oocytes

Oocytes of many species undergo parthenogenetic activation after exposure to the calcium ionophore A23187, even in the absence of external calcium (Steinhardt & Epel, 1974; Steinhardt et al., 1974), whereas those of mouse, rat and hamster will activate simply on exposure to calcium free medium (Miyamoto & Ishibashi, 1975; Whittingham & Siracusa, 1978). This indicates that calcium is involved in parthenogenetic

activation. Direct evidence of a transient increase in intracellular calcium at the time of fertilization or activation of fish (Ridgway, Gilkey & Jaffe, 1976) and sea urchin eggs (Steinhardt, Zucker & Schatten, 1977) has been obtained by using the photoprotein aequorin. Fulton and Whittingham (1978) showed that parthenogenetic activation and subsequent normal development can be induced in mouse oocytes by injecting them with calcium. When corrected for spontaneous activation, the levels of parthenogenetic activation induced in oocytes by calcium injection and by exposure to calcium free medium were 46.2% and 43.1% respectively. Fulton and Whittingham (1978) concluded that the similarity of results for the two treatments, was because either calcium free medium leads to an increase in intracellular free calcium or that activation is produced by a secondary effect common to both treatments. Magnesium injected in amounts similar to calcium failed to induce a significant level of activation, whereas strontium and barium produced effects which were not significantly different to those of calcium.

Hence this demonstration that mouse oocytes can undergo successful parthenogenetic development after activation by intracellular calcium injections, is evidence that the primary trigger for activation is probably the transient calcium flux known to occur in other species of eggs at fertilization (Ridgway et al., 1976; Steinhardt et al., 1977). In the mouse small

amounts of calcium (0.1mM) in the external medium prevent the activation of freshly ovulated oocytes (Whittingham & Siracusa, 1978). Furthermore the elevation of external calcium does not induce activation, implying that either calcium is not freely entering the oocyte or that the intracellular buffering system (e.g. mitochondria and endoplasmic reticulum) maintains a concentration of "free" calcium at a low level even if the external calcium is high.

Cuthbertson, Whittingham and Cobbold (1981) made measurements of cytoplasmic free calcium, during the artificial activation and fertilization of single mouse oocytes, injected with the calcium sensitive photoprotein aequorin. They observed the free calcium to rise exponentially from a resting level of below 0.1μM to a level greater than 5μM over a period of 10-30 minutes. A series of oscillatory calcium transients preceded this calcium rise during fertilization, but not during artificial activation. In their experiments artificial activation was induced by brief treatment (5-10 minutes) of the mouse oocyte with benzyl alcohol (a local anaesthetic) or ethanol. Such an exponential rise of intracellular calcium was also observed in eggs in which activation was induced by exposure to calcium free medium. This explains the apparent paradox that both the injection of calcium (Fulton & Whittingham, 1978) and the exposure to calcium free medium (Surani & Kaufman, 1977; Whittingham & Siracusa, 1978) activates

the oocytes. Calcium rose to 5μM in oocytes in calcium free medium with the same time course as spontaneously activated oocytes in medium containing 1.71mM calcium, suggesting that the increase in cytosolic calcium is due to the release of calcium from intracellular stores. During fertilization the calcium transients observed prior to the exponential rise had faster rising phases than the exponential rise itself. Such transients were never observed when the eggs were activated in the absence of sperm. Throughout the series of transients, a decline in the peak height and duration was observed. Experiments of Cuthbertson et al (1981) further indicated that the basal level calcium between transients started to rise towards the end of the series. This they suggest is due to the signal during transients not arising from the entire oocyte, but rather from a localized region, which may be at the site of sperm egg fusion or near to the plasma membrane of the oocyte. Similar experiments by Gilkey, Jaffe, Ridgway & Reynolds (1978) in the medaka fish oocyte (diameter about 1mm, that of the hamster and mouse oocyte is about 80μm) have shown that the intracellular calcium increase starts at the site of sperm egg fusion and then spreads around the egg.

1.12 Responses of the sea urchin egg to activation

Fertilization in the sea urchin is solely responsible for the large alterations in the metabolism of the egg which lead to the initiation of development.

The responses of the egg can be classified broadly into three groups:- morphological, physiological and biochemical (Whitaker & Steinhardt, 1982).

Morphologically the main events of activation are:-

a) cortical reaction (Moser, 1939)

b) pronuclei formation and fusion (Chambers, 1939)

c) breakdown of the nuclear envelope and chromosome condensation (Mazia, 1974) and

d) formation of the mitotic apparatus (Mazia, 1974).

The physiological reactions are:-

a) release of acid from the egg, i.e. an increased alkalinity of the egg (Johnson, Epel & Paul, 1976)

b) changes in the membrane potential (Jaffe, 1976)

c) increases in oxygen uptake (Foerder, Klebanoff & Shapiro, 1978) and

d) activation of amino acid transport systems (Epel, 1972).

The biochemical events include:-

a) initiation of protein synthesis (Grainger, Winkler, Shen & Steinhardt, 1979) and

b) DNA synthesis (Hinegardner, Rao & Feldman, 1964).

Sea urchin eggs may be activated parthenogenetically by a large number of treatments (Zucker, Steinhardt & Winkler, 1978). The link between the many different activating treatments and the many intracellular responses has been found to be simple ionic signals by which a large number of different metabolic processes are coordinated.

As mentioned previously in this chapter A23187 can activate eggs. Every parameter of normal activation by sperm is also observed when eggs are exposed to the divalent transporting ionophore. But mitosis and cleavage do not follow chromosome condensation in A23187 treated eggs (Steinhardt & Epel, 1974) - this probably being related to the absence of the sperm centriole.

Using aequorin loaded sea urchin eggs, it has been shown that the cytoplasmic free calcium concentration increases at fertilization. It reaches a peak within one minute and falls below detectable levels a minute or so later (Steinhardt et al., 1977). The peak concentration of calcium is about 5μM, assuming the calcium concentration reached is uniform throughout the cytoplasm. Addition of A23187 to aequorin loaded eggs produced a light emission with similar characteristics to that observed in response to fertilization. Removal of calcium in the sea water did not significantly change the response. The conclusion to be drawn from these experiments is that fertilization and A23187 treatment result in a transient increase in the concentration of intracellular free calcium which results from the release of calcium ions from an intracellular store. The free calcium ion concentration of the unfertilized egg is of a similar order of magnitude to that of other resting cells where it has been measured directly (Alvarez-Leefmans, Rink & Tsien, 1981).

Other parthenogenetic activators (other than A23187) also resulted in the release of intracellular calcium, except for ammonia (Zucker et al., 1978). The three classes of activators, i.e. sperm, ionophore and non electrolyte treatment are thought to release calcium in an all or none fashion from the same intracellular compartment, since treatment with any one abolished the response to either of the two, unless 40 minutes or more intervened to allow the store to "recharge". The ionophore has been found to activate eggs of many different species widely separated phylogenetically (Steinhardt et al., 1974).

Calcium ions have been shown to be the trigger for the exocytoss of cortical granules and the consequent fertilization membrane formation (Moser, 1939; Steinhardt et al., 1974). Vacquier (1975a) first showed that the isolated cortex preparation (see 1.10a), discharged it s attached cortical granules when exposed to calcium. The concentration of calcium required for discharge, in these experiments on isolated cortices has got lower, as the in vitro conditions used in these experiments has approached the ionic conditions of the cytoplasm. Steinhardt et al (1977) found values of 9-18μM, whereas only 1-3μM was required if the isolated cortical surfaces were tested with media in which magnesium - ATP was supplied and the cortices were kept in solutions of an ionic composition more closely resembling the cytoplasm

(Baker & Whitaker, 1978). A calcium concentration of 1-3 μM was sufficient to bring about discharge when calcium-EGTA buffers were rapidly introduced into eggs, by subjecting the sea urchin eggs to intense, short duration electric fields. This procedure increased the membrane permeability to low relative molecular mass substances (Baker, Knight & Whitaker, 1980). These lower levels of calcium (1-3μM) for discharge of the cortical granules found both in whole eggs suddenly lysed by electric shock and in isolated cortical fragments, correlates with estimates from aequorin loaded egg experiments (2.5-4.5μM calcium; Steinhardt et al., 1977). Further support for a calcium involvement in the cortical reaction comes from the fact that cortical granule exocytosis in intact eggs (after the addition of sperm), can be prevented by an injection of EGTA intracellularly to a final concentration of 0.25mM (Zucker & Steinhardt, 1978).

Cortical granule exocytosis is asynchronous, occurring first at the point of sperm entry, taking about 30 seconds before the cortical granules situated diametrically opposite the point of sperm entry, undergo exocytosis (Chandler & Heuser, 1979). It has been suggested that this wave of exocytosis is self propagating, rather than being the result of diffusion through the egg of a substance introduced into the egg, by the sperm (Kacser, 1955). Such an autocatalysis might be explained as a wave of calcium induced calcium release initiated by the sperm.

Shen and Steinhardt (1978) observed an increase in pH from 6.84-7.27, following fertilization in sea urchin eggs (L.pictus). These measurements were made with pH microelectrodes. The increase in pH is due to an efflux of acid at fertilization (Mehl & Swann, 1961; Johnson, Epel & Paul, 1976), both being dependent on millimolar amounts of sodium in the bathing medium (Johnson et al., 1976; Shen & Steinhardt, 1979). It is suggested that the acid efflux is due to the activation of a sodium hydrogen exchange mechanism, which follows the intracellular calcium release at fertilization (Johnson et al., 1976).

There is a marked decrease in membrane resistance following fertilization, due to the appearance of potassium channels (Steinhardt, Lundin & Mazia 1971; Steinhardt, Shen & Mazia, 1972). During ionophore activation of sea urchin eggs in zero sodium media, the calcium release occurs without the subsequent pH rise. In this situation no increase in the potassium conductance is observed (Shen & Steinhardt, 1980). Treatment with weak acids or metabolic poisons which lower the intracellular pH, can block or reverse the development of the new potassium conductance during the sensitive period when it is first appearing in the egg membrane (Steinhardt et al., 1972; Shen & Steinhardt, 1980).

1.13 Resting potentials and input resistances of eggs

The resting potential of sea urchin eggs was originally reported to be -10mV (Steinhardt et al., 1971). This value is now believed to be in error, due to a low resistance pathway created around the microelectrode during impalement of the eggs. Such a low resistance pathway ("leak") serves to depolarize the egg (Hagiwara & Jaffe, 1979). The true resting potential of sea urchin eggs is about -70mV (Jaffe & Robinson, 1978), such values being obtained only in a proportion of the eggs. This variability is caused by the extent to which the egg membrane seals around the microelectrode, and thereby reducing the leak. The greater the resistance of the leak pathway, then the closer is the measured resting potential to the true resting potential of these eggs (Chambers & de Armendi, 1979). The high resting potentials were associated with higher input resistances, of the eggs. The large negative resting potentials are similar to the values obtained by measurement of unidirectional cation fluxes (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979). The fact that the egg membrane potentials apparently fall into two groups, one at -10mV and the other at -70mV, has been attributed to properties of the potassium permeability which determines the value of the potential in the unfertilized sea urchin egg. In eggs with resting potentials of apparently -10mV, the membrane potential does not depend much on the external potassium concentration, but it is dependent on the external chloride concentration (Steinhardt et al.,

1971). When the resting potential of a sea urchin egg was found to be in the range -60 to -80mV the membrane potential was dependent on the external potassium. In the study by Jaffe (1976) the input resistances of the sea urchin eggs are reported to lie in the range 200 to 2000MΩ. The diameters of eggs of E. esculentus and P. miliaris are 150μm and 100μm respectively (Whitaker & Steinhardt, 1983).

The resting potential of the immature oocyte of the starfish, is very close to the Nernst potential determined by the internal and external potassium activities. The membrane of the immature starfish oocyte is predominantly permeable to potassium, the permeability to sodium or chloride is negligible by comparison (Hagiwara & Takahashi, 1974; Miyazaki, Ohmori & Sasaki, 1975; Shen & Steinhardt, 1976). The input resistances of mature starfish oocytes lie in the range 60 to 80MΩ in the study by Miyazaki and Hirai (1979). They reported the diameter of the oocyte to be 185 ± 10μm, (n=50).

In the annelid, Chaetopterus, the resting potential of both immature and mature eggs is about -60mV and is potassium dependent (Hagiwara & Miyazaki, 1977).

In the echiuroid, Urechis, the resting potential is about -35mV. Ion substitution experiments with these eggs indicate that the membrane is primarily potassium permeable, but has a significant sodium

permeability (Gould-Somero & Jaffe, 1977). In the study by Jaffe, Gould-Somero and Holland (1979) the mean resting potential was -33±6mV (n=133). But two of these 133 eggs are reported to have resting potentials more negative than -50mV. In the same study the input resistances are reported to be about 500MΩ.

The resting potential of fucoid algae (marine plant) eggs when measured with a single electrode were -30 to -40mV (Weisenseel & Jaffe, 1972). When a second electrode was inserted into these eggs, the membrane depolarized by about 20mV. This indicates that the electrode causes a significant leak, and hence the true resting potential is probably more negative than -40mV.

Eggs of the toad, Bufo vulgaris, isolated from the ovarian follicle into amphibian Ringer, showed resting potentials of -50 to -70mV which were potassium dependent (Maeno, 1959). Membrane potentials of the isolated oocytes of Rana pipiens (Ziegler & Morrill, 1977) and Xenopus laevis (Wallace & Steinhardt, 1977), were also measured to be near the potassium equilibrium potential. But the large potentials were only observed when the oocytes had been dissected from the follicle (called "isolated oocytes"). Unisolated oocytes (obtained following injections of hormones) showed less negative resting potentials (Wallace & Steinhardt, 1977 and Ziegler & Morrill, 1977). The increase in the negative resting potential after isolation was sensitive to ouabain and potassium free Ringer solution. It has therefore been suggested that some

fraction of the large negative membrane potential in the isolated oocyte is due to an electrogenic ion pump. Toad oocytes are reported to have input resistances varying between 200kΩand over 3MΩ (Kusano, Miledi & Stinnakre, 1977) with diameters of about 1.3mm (Grey, Bastianni, Webb & Schertel, 1982). Cross and Elinson (1980) observed resting potentials and input resistances of unisolated frog oocytes to be -28±2mV, (n=31) and 7±1MΩ, (n=21) respectively.

Values of the resting potentials recorded in mammalian eggs are summarized in Table 4 in Georgiou, Bountra, Bland, House (1984) (a copy of this paper is enclosed in Appendix D). These measurements were made on mouse, hamster and rabbit eggs. The membrane potentials of mice and hamster eggs lay in the range +2 to -35mV other than those discussed in Georgiou et al. (1983) and Georgiou et al. (1984). The results of these two studies were subsequently found to be in error and are discussed later in this thesis. At present therefore there are no published results which indicate that the resting potentials of mice and hamster eggs are close to the potassium equilibium potential, although this possibility has not been completely ruled out.

The mean resting potential of ovulated zona intact rabbit eggs is -71±2.1mV (McCulloh, Rexroad & Levitan, 1983). The average input resistance of these eggs with steady membrane potentials was 10±3.2MΩ. (Range was

2.3-56MΩ). This corresponds to a mean specific resistance of 5000Ω.cm^2 assuming a spherical egg with a diameter of 120μm.

In summary, the resting potentials have been measured in a wide variety of eggs, some of which have potassium dependent resting potentials of about -70mV. Others have smaller potentials with a relatively non-specific permeability to ions. In some cases the small potentials may be as a consequence of a leak around the microelectrode, caused by impalement of the egg. In other cases the small potentials may be real and not in error because of a leakage pathway introduced on impalement (Hagiwara & Jaffe, 1979).

1.14 Electrical events at the sea urchin egg (phylum = echinodermata; class = echinoidea) membrane during fusion of sperm and egg

The depolarization caused by fusion of a sperm and an egg has been called the fertilization potential. A fertilization potential was originally observed in eggs with resting potentials of -10mV. It had an amplitude of 10-20mV, lasting for about 1-2 minutes (Steinhardt et al., 1971). Eggs with more negative resting potentials depolarized to a similar potential at fertilization (Jaffe, 1976; Chambers & de Armendi,1979). These eggs with more negative resting potentials are electrically excitable since depolarizing current pulses elicit action potentials. Such electrically evoked potentials are quite distinct

phenomena from fertilization potentials. Chambers and de Armendi (1979) have classified the electrical events at fertilization into three separate phases. The first of these is an initial depolarization, secondly a sodium - calcium action potential and finally the sodium activation potential. Each of these is discussed below.

a) Initial depolarization

In eggs with resting potentials of -10mV, there is a slow depolarization at fertilization, which at low sperm densities can be resolved into discrete steps. These may be the first indications of sperm and egg contact (Dale, de Felice & Taglietti, 1978). These events are accompanied by decreases in the input resistance of the eggs and are observed in eggs with resting potentials of -70mV (de Felice & Dale, 1979). These workers suggest that eggs with resting potentials of -70mV are not physiological and are caused by aging of the eggs. But the work of Whitaker and Steinhardt (1983) lends support to the hypothesis that eggs with resting potentials of -70mV are indeed physiological. They were able to make extracellular recordings with suction pipettes of action currents (20-50pA, 10-20ms) which corresponded to the rising phase of the action potential. Since the threshold for the action potential is about -50mV then the resting potential must be more negative than -50mV. Furthermore such extracellular action currents were recorded in over 95%

of the eggs. The results obtained showed no correlation with time after shedding.

b) Action potential

An action potential may be elicited repeatedly in unfertilized sea urchin eggs by injecting depolarizing current pulses into the egg (Shen & Steinhardt, 1976; Jaffe, 1976; Chambers & de Armendi, 1979). The action potential is caused by an influx of calcium predominantly, and sodium. It is a component of the fertilization potential, believed to be elicited by the "initial depolarization" (discussed above; Dale et al., 1978). The action potential is not a sufficient stimulus for egg activation and activation can take place in its absence.

c) Sodium activation potential

The sodium activation potential is the third and final phase of the fertilization potential corresponding to the sodium dependent potential which persists after the action potential until the egg repolarizes. It seems to be the result of the intracellular calcium transient, since it is observed in eggs activated with ionophore (Steinhardt & Epel, 1974). Furthermore a prolonged depolarization does not serve as a stimulus to activation (Shen & Steinhardt, 1976; Jaffe, 1976). The sodium activation potential is most obvious in calcium free sea water and in eggs with resting potentials of -10mV. The potential change in calcium free sea water may be interpreted as being the fertilization potential minus the calcium dependent

action potential. This is based on the assumption that removal of calcium from the bathing medium does not alter the kinetics of the membrane permeability changes.

The absence of the calcium dependent action potential in "-10mV eggs" has been suggested to be due to inactivation of the calcium channels at this membrane potential (Jaffe, 1976).

The sodium activation potential precedes the increase in intracellular free calcium (as opposed to the transient increase in the intracellular calcium observed as a consequence of the action potential). The potential change which occurs on treatment with ionophore (A23187) is slower and smaller than that observed at fertilization (Steinhardt & Epel, 1974). The sodium activation potential may therefore be caused by sperm egg interaction. Evidence for this is that in eggs lacking the calcium dependent action potential, the sodium activation potential has superimposed on it "shoulders" which are believed to be due to individual sperm (Jaffe, 1976). Indeed in eggs of the echiuroid worm, the degree of sodium influx at fertilization is correlated with the degree of polyspermy (Gould-Somero, 1981). Furthermore interaction of single sperm have been shown to cause an increase sodium permeability over a limited portion of the echiuroid egg surface (Gould-Somero, 1981).

It is possible that the sodium activation

potential is caused by an increase in the intracellular calcium. Dale and de Santis (1981) have shown that the duration of the sodium activation potential is determined by the duration of the cortical reaction. The membrane conductance change may be brought about by either a direct action of the increased cytosolic calcium or as a consequence of cortical granule exocytosis. The sodium activation potential declines soon after the cortical reaction (Chambers & de Armendi, 1979), quite abruptly to about zero mV, then more slowly until the resting potential of the fertilized egg stabilizes at about -70mV, about 5 minutes after fertilization. The slow decrease is suggested to be due to the increase in the potassium conductance of the egg membrane caused by the increase in the intracellular pH (Shen & Steinhardt, 1980).

In summary, the initial sperm dependent depolarization elicits a calcium dependent action potential. The falling phase of the action potential overlaps the rising phase of the sodium activation potential caused by the increased sodium conductance. The slow repolarisation to -70mV is due to the resequesteration of calcium and the pH dependent increase in potassium conductance.

The membrane potential itself is not important in terms of development, since eggs may be held at 0mV without activation occurring (Jaffe 1976) or at the resting potential during activation without development

being affected (Mackenzie and Chambers, 1977). But the potential change does decrease the probability of more than one sperm fusing with an egg (as discussed in the next section).

1.15 The block to polyspermy in sea urchin eggs

The mechanisms employed by the egg to reduce the probability of entry of supernumerary sperm is known as the block to polyspermy. This is a composite process. A fast but incomplete block has been described which begins about 3secs after fertilization and reduces the probability of subsequent sperm entry to 5% of the initial value (Rothschild & Swann, 1952; Presley & Baker, 1970). A complete block to polyspermy is present 30-60 seconds after fertilization and coincides with the elevation of the fertilization membrane. The "fast block" might correspond to the reduction of sperm receptors on the egg membrane due to the release of protease (present in cortical granules). After the development of a complete block the egg may be refertilized by removal of the fertilization membrane. The fast block is electrical in nature, since it was not possible to activate eggs voltage clamped at +10mV (Jaffe, 1976). Hence the action potential which is a component of the fertilization potential prevents sperm fusion.

1.16 Fertilization potential in starfish

(Phylum=echinodermata; class = asteroidea)

The starfish oocyte (Asterinia pectinifera) is fertilizable at any stage of maturity after germinal vesicle breakdown. But the optimal period (eggs in this period have been called mature eggs) for fertilization resulting in normal development is between breakdown of the germinal vesicle and the appearance of the first polar body (Miyazaki & Hirai, 1979). Monospermy occurs in more than 90% of starfish oocytes when they are fertilized in this period. Eggs fertilized after this period tend to become polyspermic (frequency of monyspermy decreases). Indeed "over-ripe" oocytes inseminated after formation of the second polar body, usually become polyspermic, even though a normal fertilization membrane is formed. This has been attributed to the absence of a fast electrical block in over-ripe eggs. In mature oocytes the peak of the fertilization potential exceeded -5mV. This has been called the critical potential level for the fertilization block by Miyazaki and Hirai (1979). This is because sperm entry into the starfish oocyte was prevented when the egg was voltage clamped at potentials more positive than -5mV, and multiple sperm entries induced when the potential was more negative. The rise time of the fertilization potential was less than 1 second and the potential stayed at +12mV even after the initiation of fertilization membrane elevation. Since the potential after fertilization is more positive than the critical potential level, the

entry of further sperm is prevented. Whereas in over-ripe eggs the fertilization potential took about 15 seconds to reach -5mV and in some eggs this critical potential was not reached after fertilization. In over-ripe oocytes the multiple sperm entries, were associated with step depolarizations on the fertilization potential before membrane elevation took place. Thus in over-ripe eggs it was possible for second and further sperm to fuse with the egg during the 15 second period required for the potential to reach -5mV; i.e. there is no fast electrical block in over-ripe eggs. No precise observation has been made for the timing of the cortical reaction, but since the shortest time for the initiation of the fertilization membrane elevation is about 40 seconds after the arrival of sperm at the oocyte, it is considered by Miyazaki and Hirai (1979) that the fast polyspermy block is required for at least 40 seconds after sperm attachment.

1.17 Fertilization potential in the marine worm (phylum = echiuroidea)

The fertilization potential of the marine worm (Urechis caupo) is similar to that observed in sea urchins. The membrane potential shifts in a step from the unfertilized resting potential of about -30mV to a peak potential near +50mV, with a rise time of about 1 sec (Gould-Somero & Jaffe, 1977; Jaffe, Gould-Somero

& Holland, 1979). The most rapid responses occur within 2 seconds after insemination, longer delays resulting from the time required for the sperm to reach the egg. The potential falls to about +30mV and finally becomes negative again, reaching the unfertilized egg resting potential at about 10 minutes then continuing to -60mV at about 20 minutes.

In the Urechis, the fertilization potential is large and positive, long lasting and uniform amongst individuals. Since there is little if any cortical granule exocytosis during the first 40 minutes after insemination (Gould-Somero and Holland,1975), detailed ionic studies have been performed on this preparation (Jaffe et al., 1979). Ion substitution and tracer flux experiments indicate that the fertilization potential is due to a large increase in sodium permeability, and a small increase in calcium permeability during the early phase. The maximum amplitude of the fertilization potential ignoring the first 15 seconds of the response, shows a 50mV slope for a tenfold change in the extracellular sodium (choline substituted). This "phase" was independent of calcium in the bathing medium. But the first 15 seconds of the response is dependent on both external sodium and calcium. During the response the sodium and calcium influxes increased to 20-30 times those of the unfertilized egg but the sodium influx is about 15 times larger than that of the calcium. An acid efflux also occurs (Paul, 1975). An increase in potassium

permeability was also observed (smaller than in sea urchin), but it is insufficient to account for the termination of the fertilization potential.

A response similar to the fertilization potential (activation potential) with the associated ion fluxes can be elicited by trypsin treatment of the Urechis egg. It can therefore be concluded that the sodium and calcium channels pre-exist in the egg membrane. As mentioned previously in this chapter the sodium influx is "sperm gated", being proportional to the number of sperm entries. But the calcium influx is near maximal after a monospermic fertilization. The calcium influx is via voltage activated channels, since calcium action potentials are elicited by depolarizing pulses. The calcium influx in 50mM sodium sea water is less than that in normal sea water containing 500mM sodium. This is because the amplitude of the fertilization potential is less in 50mM sodium sea water than in 500mM sodium sea water and the peak is more negative than the threshold for the calcium action potential. Depolarizing pulses do not elicit a sodium influx hence the sodium channels are not voltage activated.

1.18 Fertilization potential in tunicates (phylum = chordata; class = ascidiacea)

Resting potentials of ascidian oocytes (Ciona) were observed to fall into two categories (Dale, de Santis & Ortolani, 1983). One group had low resting

potentials in the range -20 to -35mV and another had high resting potentials falling in the range -80 to -90mV. The two groups when fertilized, developed normally, although the fertilization potential was different in the two cases. Fertilization of high resting potential oocytes elicited a rapid step depolarization (fertilization potential) with a rise time of less than 0.2 seconds and a peak of about +10 to +20mV. The potential then remains at about zero level for several minutes and then slowly repolarizes. Whereas in oocytes with low resting potentials, the fertilization potential was slower (rise time about 2 to 3 seconds) and overshot by only 2 to 5mV. In these low resting potential oocytes, the fertilization potential slowly repolarizes to reach the original resting potential after 1 to 2 minutes (Dale et al., 1983). Polyspermic fertilization was induced by (a) removal of the extracellular coats - "chorion", (b) aging the oocytes and (c) using high sperm densities. The fertilization potentials in monospermic and polyspermic eggs were found to be the same (Dale et al., 1983). Additional sperm interactions did not give rise to further electrical events.

The conclusion drawn from these experiments is that the plasma membrane of ascidian oocytes lacks intrinsic mechanisms to prevent polyspermy. It has been shown (Rosati & de Santis, 1978; Rosati, de Santis & Monroy, 1978) that in Ciona the chorion is responsible for the prevention of polyspermy. This may

be related to the fact that ascidian oocytes lack cortical granules. Another interesting feature of the study by Dale et al (1983) was that they observed spontaneous action potentials in 70% of the oocytes with high resting potentials. These action potentials consisted of a rapid depolarization overshooting zero by 30-40mV, repolarizing partially to a plateau of about +10mV for 10-60 seconds and then the potential returns to its original value. They indicated a seasonal variation in the frequency of spontaneous action potentials observed in eggs and suggested that this may be related to oocyte maturity. Furthermore such spontaneous action potentials in high resting potential oocytes were much more frequent following insemination. But there was no correlation between a spontaneous action potential and a sperm entry.

Embryos of tunicates have been used extensively for investigating changes in the membrane properties during development. Takahashi & Miyazaki (1971), Miyazaki, Takahashi & Tsuda (1972), Okamoto, Takahashi & Yoshii (1976a,b) and Takahashi & Yoshii (1978,1981) have studied membrane excitability in two species of tunicates - Halocynthia roretzi and Halocynthia aurantiem. These authors observed excitability in the form of anode break responses (elicited by hyperpolarizing pulses) in eggs and embryonic cells up to the beginning of invagination (128 cell stage). The cells had resting potentials of -19.4±7mV and did not

exhibit spikes in response to depolarizing current stimuli or spontaneous activity. A transition to a higher resting potential occurs at the 128 cell stage, at which time action potentials are observed in response to depolarizing current pulses. In another species of tunicate (Clavelina huntsmani), two groups of resting potentials have been recorded in eggs and some embryonic cells. One group had potentials between -20 and -30mV and the other between -60 and -75mV (Thompson & Knier, 1983; however, in three records of the resting potential shown in their study, the values were close to -50mV). In some of these embryonic cells conversion from one resting voltage to the other was possible by stimulation with hyperpolarizing or depolarizing current pulses (about 1nA and 5 seconds). Embryonic cells with resting potentials in the high range (-60 to -75mV) for example could be converted to eggs with a resting potential in the low range (-20 to -30mV) by application of a short depolarizing pulse. Similarly it was possible to convert a "low potential cell" to a "high potential cell" by stimulating with a short hyperpolarizing pulse (Thompson & Knier, 1983). Furthermore these eggs with high resting potentials fired action potentials in response to depolarizing pulses. In these embryonic cells spontaneous action potentials and trains of spontaneous action potentials were observed. Spontaneous action potentials were not correlated with any of the other changes that occurred in the fertilized egg.

The membrane potential during both electrically evoked and spontaneous action potentials sometimes did not return to the original more negative resting potential and remained within a more depolarized range for an indefinite period. In such embryonic cells the resting potential could be "reset" to the original high potential range by a short hyperpolarization. Some low potential cells suddenly and rapidly repolarized to a high potential state (Thompson & Knier, 1983). These workers have suggested that if the multicellular embryo was comprised of two groups of cells with different resting potentials then:-

a) high resting potentials allow the expression of spontaneous action potentials, which are due to transient influxes of sodium and calcium. The concentration of sodium and calcium intracellularly in the group of cells with high potentials may modulate various metabolic processes and

b) regional differences in the resting potential may lead to the establishment of gradients of ionic current flow between blastomeres.

1.19 Fertilization potential in amphibians (phylum = chordata; class = amphibia)

A fertilization potential has been recorded in eggs of Rana pipiens (frog). It consists of a shift of the membrane potential from a resting value of -28mV to +8mV in a single step lasting less than 1 second (Cross

& Elinson, 1980). A second slower shift was superimposed on this reaching a maximum amplitude of +17mV. The membrane potential remained positive for a total of 21 minutes. When the membrane potential of unfertilized eggs exposed to sperm, was current clamped at potentials between +1 and +22mV, for 30 minutes then the initiation of the first cleavage furrow was delayed for 20 minutes. This indicates that the egg was not fertilized while the membrane potential was positive (Cross & Elinson, 1980). It is interesting that in 8 of the recordings from unfertilized eggs by Cross and Elinson (1980), resting potentials in the range -50mV to -67mV were observed.

Maeno (1959) noted a positive shift in the membrane potential when the Bufo egg (toad) was activated (initiation of development) by electrode penetration. In tap water, this activation potential consisted of a positive shift in the resting potential from about -30mV to a peak potential of +50mV. The shift in the potential began about 30 seconds after electrode penetration, the peak potential was reached within 1 or 2 minutes. The potential then declined gradually reaching a steady state near 0 mV after about 5 minutes. Resistance measurements indicated an approximately 20 fold increase in the membrane conductance (due to a chloride permeability increase - peak voltage shifts about 56mV for a 10 fold change in the external chloride concentration) during the activation potential.

The change in input resistance was from 7MΩ at the onset of the fertilization to 0.3MΩ, measured 2 to 5 minutes after the onset of the fertilization potential (Cross & Elinson, 1980).

Ion substitution experiments also indicated an electrical block to polyspermy in Rana pipiens (Cross & Elinson, 1980). For example 25% of the eggs fertilized in the presence of 40mM sodium iodide, became polyspermic. In this solution the peak amplitude of the fertilization potential was -20mV (i.e. below the critical potential level of about +1mV). Fertilizations in the presence of 40mM sodium chloride were not polyspermic (in this solution the shift in the fertilization potential was the same as that in 10% Ringer, the latter being the standard saline solution used).

The fertilization potential of the frog egg provides a fast transient block to polyspermy (Cross and Elinson, 1980; Grey et al, 1982; Charbonneau, Moreau, Picheral, Vilain and Guerrier, 1983). For Rana pipien eggs, the amplitude of the fertilization potential changes by 48mV for a ten fold change (actual change from 40 to 113mM) in the external chloride (Cross, 1981). This indicates that some other ionic conductance is significant during fertilization. Studies on R. pipiens (Cross, 1981) and R. temporaria (Charbonneau et al, 1983) indicate that sodium is not a major current carrier during fertilization. The role

of a potassium conductance during the fertilization or activation potential was investigated by Ito (1972), who found that the activation potential amplitude changed only slightly when the solution was changed from one containing 113mM sodium to one containing 113mM potassium. But in both of these solutions the peak of the activation potential was at a negative voltage. Recently Jaffe and Schlichter (1985) have investigated the potassium contribution to the fertilization potential, in solutions with lower chloride concentrations (because in such solutions the fertilization potential would have been positive). They observed that the fertilization potential involves two separate conductances i.e. chloride and potassium. The equilibrium potential of the fertilization current (+3mV) is between the chloride (+18mV) and potassium (-160mV) equilibrium potentials, although closer to the former. They observed that the conductance increase preceded the capacitance increase by several seconds. Therefore the initial appearance of chloride and potassium channels cannot be accounted for by the addition of the limiting membrane of cortical granules. A similar conclusion has been reached for the sea urchin egg, since in that the fertilization potential precedes the cortical reaction (Jaffe et al 1978), and for the tunicate egg, in which the surface area of the egg membrane is constant during the activation potential (Kozuka and Takahashi, 1982).

The slower, long lasting polyspermy block in

mature frog oocytes is due to the fertilization membrane formed following cortical granule exocytosis (Grey, Working & Hedrick, 1976). Results of Schlichter and Elinson (1981) suggest that an increase in intracellular calcium may cause electrical events that function as a fast block to polyspermy. Treatment with A23187 produced a rapidly rising depolarization that usually remained positive long enough to function as a fast block to polyspermy. More direct evidence for the role of calcium is that iontophoretic injection of calcium into mature Rana pipiens oocytes, can elicit an activation potential, which is not unlike the fertilization potential observed in these eggs (Cross, 1981).

In eggs of Xenopus laevis, the resting potential is -19mV (these were "unisolated oocytes", i.e. ovulation had been induced by subcutaneous injection of HCG) and fertilization triggers a rapid depolarization to +8mV (i.e. the fertilization potential). The potential remains positive for about 15 minutes (Grey, Bastiani, Webb & Schertel, 1982). Activation of the eggs with A23187 produces a slower but similar depolarization. As in other amphibian eggs discussed above, the fertilization potential (and the activation potential elicited by treatment with ionophore) results from a net efflux of chloride, since the peak of the fertilization potential decreases as the concentration of chloride in the medium is increased. As in the ion

substitution experiments discussed above with reference to Rana pipiens (Cross & Elinson, 1980), Xenopus also became polyspermic in solutions in which the chloride is replaced by iodide (Grey et al., 1982). The resting potential of Xenopus eggs ("unisolated oocytes") in 27% DeBoer's saline containing 20mM sodium iodide (27% DeBoer's saline is the standard saline used for Xenopus eggs) was about -16mV. In this medium the fertilization potential consisted of a hyperpolarization to -21mV, i.e. the fertilization potential was reversed. Similarly a hyperpolarization occurred in the same medium when the eggs were activated with A23187. This lends further support to the electrical block to polyspermy, since the membrane potential of the newly fertilized or activated egg is more negative than the resting potential (Grey et al., 1982). Since the iodide containing solutions altered the fertilization potential (fast block) but did not interfere with the formation of the fertilization membrane (the slow block to polyspermy) the Xenopus system was used to investigate the physiological significance of the fast electrical block to polyspermy (Grey et al., 1982). Fertilized eggs were obtained from experiments in which the mating pairs deposited eggs in 20mM sodium iodide in 27% DeBoer's saline. The results of these experiments showed that significant levels of polyspermy occur at physiological concentrations of sperm, in the absence of the electrical block imposed by the plasma membrane.

Acetylcholine causes a selective increase in the chloride permeability of toad oocytes (Kusano et al., 1977; Kusano, Miledi & Stinnakre, 1982), in a dose dependent manner, with threshold concentrations as low as 10^{-9}M. The reversal potential of the acetylcholine induced response was about -19mV, which is in close agreement with the equilibrium potential for chloride (intracellular chloride measured with a chloride ion sensitive microelectrode, was found to be 65mM). Robbins and Molenaar (1981) have tested the hypothesis that cholinergic mechanisms play a role in fertilization. They concluded that such a role was unlikely, but suggested that a transient release of acetylcholine which occurs on immersion of eggs into hypotonic solutions (simulating events during natural oviposition), may be biologically significant.

1.20 The mouse has no electrical block to polyspermy

Braden, Austin & David (1954) noted that during natural mating only 1.2% of mouse eggs were polyspermic, whereas in 17.4% of the eggs two or more sperm had passed through the zona pellucida. Since all the eggs in which the zona was penetrated, eventually contained at least one sperm in the cytoplasm (Braden & Austin, 1954), the results indicated that in about 16% of the eggs a second sperm had been excluded from the cytoplasm, by a plasma membrane block or by cytoplasmic elimination. Subsequent in vitro studies indicated

that a major component of the post-zona block was at the level of the egg plasma membrane (Pavlok & McLaren, 1972; Wolf, 1978; Yu & Wolf, 1981). The possibility that this might be an electrical block was investigated by Jaffe, Sharp & Wolf (1983). They observed that starting from an unfertilized value of -41±4mV, the mean membrane potential underwent an oscillation of 4±1mV in amplitude, starting 7±5 minutes after insemination, and lasting for about 1 minute. But other than this small oscillation, the membrane potential remained constant during 60min of recording following insemination. The range of membrane potentials during this period of recording was 11±4mV, which was not significantly different from that which was observed in 60 minute recordings from unfertilized eggs (Jaffe et al., 1983). They observed that as a consequence of fertilization, the resistance of the membrane decreased from 96±34MΩ to 44±15MΩ.

In unfertilized eggs, the resistance at the start of the recording was occasionally as high as 200MΩ, but was usually about 100MΩ. But the membrane resistance of unfertilized eggs declined steadily, falling to half its initial value of about 100MΩ, in 22 minutes (compared to 4 minutes for fertilized eggs). They interpreted the initial high values as being the most natural, and the decrease in resistances as being artifactual, since insertion of a second microelectrode in the same egg always caused a further decrease in resistance. In their study they did not use zona

intact eggs, because in these, the time delay between insemination and contact of the fertilizing sperm with the egg plasma membrane (33-85 minutes; Sato & Blandau, 1979) is too long. Hence the membrane resistance of the egg would have decreased seriously between the time of electrode insertion and the probable time of fertilization.

Jaffe et al (1983) speculate that the voltage oscillation at fertilization may be a consequence of sperm egg fusion (since all times quoted are those after insemination, no indication being given of the instant of fusion), or cortical granule exocytosis, or possibly analagous to the recurring hyperpolarizations observed in hamster (see 1.21) and rabbit (see 1.22) eggs (Miyazaki & Igusa, 1981, 1982; McCulloh, Rexroad & Levitan, 1981).

Jaffe et al (1983) also performed experiments in which they varied the concentration of sodium or calcium in the bathing solution. Since if the mouse did have an electrical block to polyspermy, and if the fertilization potential was produced by a permeability increase to sodium or calcium (amongst other ions), then varying the ionic composition might induce polyspermy, as is observed in other species (Gould-Somero, Jaffe & Holland, 1979). Their results indicated that the average number of fertilizing sperm per egg is not increased by a reduction of the sodium from 168-26mM or a reduction of calcium from 5.0-0.5mM.

But the interpretation of such results, is complicated by the possibility that the altered ionic conditions, might affect many steps in the fertilization process. For example in 26mM sodium solution they observed that the number of sperm per egg was decreased. This may be explained by a deleterious effect of low sodium on the sperm. Their conclusion was that lowering sodium or calcium did not impair the block to polyspermy. The study of Jaffe et al , (1983), therefore indicated that the mouse does not have an electrical block to polyspermy since no shift in the membrane potential was observed at fertilization (with the exception of a small oscillation, the amplitude and duration of which are so small that they are unlikely to be physiologically significant).

In another study by Igusa, Miyazaki & Yamashita (1983), homologous fertilizations of mice eggs elicited small hyperpolarizations (3-4mV) superimposed on a gradual hyperpolarization, from -35 to -55mV, during 50mins of intracellular recording after fertilization. Both the hyperpolarizing responses and the hyperpolarizing shift were associated with an increase in the membrane conductance. The reversal potential of the hyperpolarizing responses was found to be about -80mV, indicating that they were probably due to an increased potassium conductance. Since iontophoretic injection of EGTA into the fertilized eggs abolished the hyperpolarizing responses, and since they were not affected by removal of chloride from the bathing

medium, it is suggested that they are due to a calcium activated potassium conductance (Igusa et al., 1983).

In unfertilized mouse eggs no significant increase in the potassium conductance was induced by calcium injection (occasionally a small hyperpolarization of 3-4mV was produced, but associated with an increase in the membrane resistance, apparently due to a decrease in the leakage current around the microelectrode) with currents up to 4nA, whereas a 0.4nA pulse caused a substantial increase in the potassium conductance in unfertilized hamster eggs (see 1.21) (Igusa & Miyazaki, 1983). In the study by Igusa et al.(1983), the resting potential and the input resistance of the mouse eggs was -35.0 ± 5.9mV, (n=26) and 140 ± 47MΩ, (n=19) respectively. The first hyperpolarizing response occurred 9-12mins after the cessation of flagellar motion of the sperm (an indicaton of the instant of sperm penetration - see Chapter 2). The hyperpolarizing responses occurred thereafter at intervals of 2-6 minutes. The hyperpolarizing responses were observed until at least 2 hours after fertilization.

1.21 Fertilization potential in golden hamster eggs consists of recurring hyperpolarizations

Fertilization of golden hamster eggs elicits recurring hyperpolarizations (Miyazaki and Igusa, 1981, 1982). Each hyperpolarizing response during

fertilization, was found to be caused by an increase in the potassium conductance, activated by an increase in the intracellular calcium concentration. This conclusion by Miyazaki and Igusa (1982) is based on the following observations:-

a) The reversal potential of the hyperpolarizing response, shifted according to the Nernst equation for potassium ions, when the external potassium concentration was changed from 5mM to 20mM, whereas it was unaltered by the removal of chloride ions.

b) The hyperpolarizing response was blocked by the intracellular injection of EGTA and

c) Injection of calcium into an egg induced a hyperpolarization of the membrane similar to the hyperpolarizing response (this has also been observed by Georgiou et al., 1983).

The hyperpolarizing response associated with sperm entry into the egg occurred at any potential between -160 and +50mV. Hence a potential dependent block of sperm entry does not occur in the hamster egg (Miyazaki & Igusa, 1982). The resting potential of unfertilized hamster eggs in the study by Miyazaki & Igusa, (1982) was -29 ± 6mV , (n=160) and the input resistance was 150 ± 66MΩ,(n=135). They noted that the more negative resting potentials were associated with the larger input resistances, suggesting that there was a substantial leakage due to electrode impalement in most cases. The largest resting potential observed by them was -50mV with a membrane resistance of 400MΩ (Miyazaki

& Igusa, 1981).

The duration of hyperpolarizing responses induced by calcium injection and those evoked by fertilization were 10.1±1.9 seconds, (n=60) and 11.3±2.3 seconds, (n=300) respectively. The reversal potential of the hyperpolarizing response evoked by fertilization was -82.7±2.2mV (n=34) in a bathing solution containing 5.5mM potassium (Miyazaki & Igusa, 1982).

The hyperpolarizing responses induced by fertilization are superimposed on a gradual hyperpolarizing shift of the resting potential (Igusa & Miyazaki, 1983). They showed that the hyperpolarizing responses were abolished by the removal of external calcium. Their frequency was decreased by lowering the calcium in the bathing medium or by adding manganese or cobalt, and it was increased by raising the calcium in the bathing medium. The frequency of the hyperpolarizing responses was decreased during a sustained depolarization by injecting steady depolarizing (outward) current. The frequency was increased during a steady hyperpolarization. But the amplitude, conductance increase, and the reversal potential of each hyperpolarizing response was hardly affected by the concentration of calcium in the bathing medium, or by the presence in it of either manganese or cobalt. The hyperpolarizing shift was decreased by lowering the calcium in the bathing medium (this may be partially explained by the reduced stabilizing effect

of calcium at lower concentrations), by adding manganese or cobalt to the bathing medium, or by the injection of EGTA. Thus it was concluded by Igusa & Miyazaki (1983) that the hyperpolarizing shift may reflect a continuous calcium influx stimulating a calcium activated potassium conductance.

When the injection current of calcium was increased slowly in a stepwise manner, the hyperpolarizing response became larger and eventually, a much larger hyperpolarization was evoked at a "critical injection current" (1.10±0.22 nA, n=55). This enhanced response has been called a "full response" by Igusa and Miyazaki (1983). Once a full response occurred the same threshold pulse failed to generate a second full response for 1-2 minutes, but instead a smaller hyperpolarizing response was produced, which started to decay as soon as the injection pulse ended. The recovery of the full response was not obtained until at least 60 seconds after the end of the previous full response. This period has been called the "refractory period".

In impaled fertilized eggs, a hyperpolarizing response could be induced, by a calcium injection with a smaller pulse than necessary in unfertilized eggs. In fertilized eggs the refractory period was shortened to 40-50 seconds which is comparable to the interval between successive hyperpolarizing responses elicited in polyspermic eggs. This extensive study by Igusa and Miyazaki (1983), indicated that the hyperpolarizing

responses at fertilization, required the presence of external calcium and that the frequency of the hyperpolarizing responses was dependent on the concentration of calcium in the bathing medium. No depolarizing phase was observed to precede any of the hyperpolarizing responses. It is concluded that the increase in intracellular calcium (which is responsible for activating the potasium conductance, and hence causing the hyperpolarizing response), reflected in the hyperpolarizing response is caused directly by a transient enhancement of calcium influx, but is mediated by intracellular processes such as calcium release from stores. The recovery phase of the hyperpolarizing response, is considered to reflect the removal of the increased intracellular calcium. The cytosolic calcium concentration may be restored to it's "resting level" by calcium buffering systems, or by calcium extrusion from the egg (eg by a sodium calcium exchange). One of the factors determining the refractory period is probably the time required for calcium stores to replenish. Support for this comes from the prolongation of the refractory period upon lowering the temperature. At lower temperatures (26^{o}C compared to 31^{o}C in the rest of the experiments), a full response was elicited more easily, probably due to the reduced calcium buffering power. Furthermore in calcium free medium, complete recovery of the full response required more than 3 minutes in unfertilized

eggs, suggesting that the calcium supply from the outside is an important factor in reloading the calcium stores. Another factor determining the refractory period may be a rise in the threshold or inactivation of calcium induced calcium release.

The critical injection current, required to elicit a full sized hyperpolarizing response in fertilized eggs, was about one tenth that in unfertilized eggs. One of the reasons for this may be that the threshold for the calcium induced calcium release, is lower in fertilized eggs, than in unfertilized eggs. The fact that no hyperpolarizing responses are elicited by fertilization in calcium free solutions, is an indication that either a calcium influx or the binding of calcium with sites on the outer membrane surface is required. Although calcium free bathing solutions may be harmful to the egg, generally. The results of Igusa and Miyazaki(1983) indicate that a calcium influx is required. Therefore it seems probable that the interval of the periodic increase in intracellular calcium, is determined by the balance between the loading of calcium stores in the egg, and the basal level of intracellular calcium as an inducer of calcium release. The calcium influx is capable of determining the interval between the fertilization evoked hyperpolarizing responses, by changing the rate of accumulation of calcium in the stores.

Hamster eggs fertilized with mouse sperm (MsxHe) also showed transient recurring hyperpolarizations,

superimposed on a hyperpolarizing shift of the resting potential, which gradually reached -60mV approximately (Igusa et al., 1983). Unlike the hamster sperm, the cessation of flagellar motion of the first mouse sperm failed to induce the first hyperpolarizing response, but produced only a small hyperpolarizing "step" of 3-7mV. Similar steps occurred for each additional sperm with a one to one correspondence, 4-50secs before the cessation of sperm motion. In hamster eggs fertilized with mouse sperm the first hyperpolarizing response appeared about 15 minutes after the cessation of flagellar motion of the first sperm (this is an indication of the instant of fusion between sperm and egg - see Chapter 2). The intervals of the hyperpolarizing responses thereafter were in the range 2-10 minutes, in contrast to 30-45 seconds in hamster eggs inseminated with hamster sperm (HsxHe). The hyperpolarizing responses in MsxHe eggs, were abolished by intracellular injection of EGTA, suggesting that they were caused by a periodic increase in the intracellular calcium concentration, as in hamster eggs. Histological observations reveal that the resumption of the second meiosis, the indication of egg activation, is delayed in MsxHe eggs by about 15 minutes compared with that in HsxHe eggs. There was a good correlation between the delay of activation and that of the occurrence of the first hyperpolarizing response. Eggs in which sperm penetration was not

followed by activation, showed no hyperpolarizing responses (Igusa et al., 1983). The amplitude of the hyperpolarizing step elicited by successive sperm penetrations, gradually decreased until it became undetectable. This may be due to the membrane potential approaching the equilibrium potential for potassium ions and /or to the progressive decrease in the membrane resistance.

1.22 Fertilization potential in rabbit eggs consists of a slow depolarization, superimposed on which are repetitive diphasic membrane potentials

As mentioned previously in this chapter, the plasma membrane of the rabbit egg allows only one sperm to enter the egg during fertilization. Work by McCulloh et al (1983) has shown that ovulated zona intact eggs of the rabbit, have a mean membrane potential of -71 ± 2.1mV. They report that a stereotypic response occurred 12-135 minutes following insemination, which consisted of a slow transient depolarization (8 ± 1.5mV) upon which were superimposed up to 36 repetitive "diphasic insemination potentials" (DIPs). Each of these DIPs consisted of a brief hyperpolarization (2.0 ± 0.44mV which lasted 8.7 ± 0.44 seconds) followed by a depolarization (2.5 ± 0.45mV). The stereotypic responses recorded in zona free eggs, were similar in amplitude and timing to those observed in zona intact eggs.

CHAPTER 2 GENERAL METHODS

2.1 Isolation and preparation of eggs

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2.2 Isolation and preparation of sperm and the subsequent insemination of eggs

a) Golden hamsters b) Mice

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2.4 Solutions

a) The composition of normal bathing solution

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e) Abbreviations used throughout the text to describe the composition of bathing solutions

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g) The composition of mouse capacitation medium

2.5 Visual observation of fusion

2.6 Histological confirmation of fertilization

2.7 Control clamp experiments

2.8 Do electrically evoked action potentials activate zona-free hamster eggs?

2.9 Controls for the fertilization experiments

2.10 Summary

In this chapter, experimental protocols which have been used throughout the course of this study are described, i.e. the preparation of eggs and sperm, recording techniques and finally how the eggs were examined to confirm fertilization.

2.1. Isolation and preparation of eggs

Eggs used during this study were obtained from two different species of animals:-

a) Golden Hamsters

b) Mice

The procedure for the isolation of eggs was the same in both species although the subsequent preparation was slightly different.

a) Golden Hamsters

The animals (6-12 week old) were maintained under a fixed light/dark cycle (16 hours light/8 hours dark). Superovulation was induced by injection (I.P.) of 30i.u. Pregnant Mare Serum Gonadotrophin (Folligon, Intervet Labs Ltd., Cambridge) in the early evening, followed by an injection (I.P.) of 45 i.u. Human Chorionic Gonadotrophin (Sigma Chemical Co., St. Louis, U.S.A) 48 hours later.

Animals were killed 13-15 hours after the second injection by cervical dislocation, and their oviducts were placed in a dish containing a physiological solution (called normal in this text, the composition of which is given in 2.4a). Eggs were removed from the

tubal ampullae and placed in the normal solution, containing hyaluronidase (1mgml^{-1}, Type 1-S Sigma) for about 1 minute, to remove the cumulus oophorus. To remove the zona pellucida each egg freed from the cumulus was bathed for 1-3 minutes in normal solution containing trypsin (1mg ml^{-1}, Type III, Sigma). All enzyme treatments were carried out at room temperature. The cumulus oophorus free eggs were then washed twice, in normal solution and stored in an incubator at 37^{o}C. Trypsinization was performed on individual eggs as required.

During trypsinization the removal of the zona pellucida was monitored with a microscope (x40). For experiments in which recordings were performed with intracellular electrodes, the egg was removed from the trypsin solution as soon as the zona pellucida had visibly come off the egg. For recordings with patch pipettes the eggs were allowed to remain in the trypsin solution for a further 45-60 seconds. This was an attempt to "clean" the egg membrane further and hence facilitate giga-seal formation. For those experiments in which it was necessary to have an intracellular recording electrode as well as a patch pipette sealed to the surface of the egg, the "extended" trypsin treatment was used. This was the case even if such a dual recording system was to be used to monitor the electrical events during fertilization (since such an extended trypsin treatment did not inhibit sperm egg

fusion in hamster eggs). The eggs in every experiment were washed three times with normal solution after treatment with trypsin.

Cumulus-oophorus free eggs occasionally underwent spontaneous activation during incubation for more than 1.5 hours, and released the second polar body. Such eggs were discarded.

b) Mice

Details of obtaining zona-free eggs from mice were similar to those described for hamsters, with the following exceptions. Animals were injected (I.P.) with 10 i.u. PMSG in the early evening and with 10 i.u. HCG 48 hours later. To remove the cumulus oophorus each egg was incubated for five minutes in normal solution containing hyaluronidase (1mg ml^{-1}). To remove the zona pellucida each egg (freed from cumulus) was bathed for 5-8 minutes in normal solution containing protease (2mg ml^{-1}, Type XIV, Sigma).

The main aim in these experiments on mice eggs was to study single channel currents, for which it was necessary to have a high resistance seal, between the patch pipette and the egg membrane. Formation of such high resistance seals was found to be facilitated by an extended protease treatment of the membrane (see 2.3b).

2.2 Isolation and preparation of sperm and the subsequent insemination of eggs

The majority of fertilization experiments

described in this study, were homologous fertilizations of hamster eggs (Chapters 5,6,7 and Appendix A). A small number of heterologous fertilizations of hamster eggs were performed with mice sperm (Chapter 5). Studies of other workers have shown that zona-free hamster eggs, may be fertilized with species of sperm other than the hamster (Hanada and Chang, 1972; Yanagimachi, 1972b). No attempts were made to fertilize mice eggs with hamster sperm.

The preparation of each specie of sperm for fertilization experiments was completely different:-

a) Golden Hamsters

Male golden hamsters older than 10 weeks, weighing a minimum of 100g, were used as sperm donors. Both cauda epididymes were dissected from the animal, then any fat and blood vessels were removed from their surface. The surface was further cleaned using filter paper moistened with double distilled water. The surface membrane of the cauda epididymis was cut, such that two or three of the sperm filled tubules within were exposed. Each cauda epididymis was then placed near the edge of a sterile plastic dish, filled with liquid paraffin. Each dish also contained a centrally placed droplet of modified Tyrode solution (the composition of which is given in 2.4f), such that the liquid paraffin completely covered it. Both the modified Tyrode and the liquid paraffin had previously

been equilibrated with 5% CO_2 95% air (because Tyrode solution is buffered using bicarbonate).

It was necessary to ensure that all the instruments were kept warm (using a hot plate and/or an incubator, both being held at 37°C).

A small nick was then made in one or two of the exposed sperm filled tubules, causing a tiny droplet of sperm to be released into the liquid paraffin. The size of this droplet (i.e. the number of sperm to be isolated) could be increased by applying gentle pressure to the cauda epididymis using a coarse pair of forceps. This droplet of sperm was then drawn into the droplet of Tyrode solution placed in the centre of the dish using a fine sterile glass hook. The sperm then swam freely away from the glass hook, to distribute themselves evenly within the Tyrode solution.

The concentration of sperm isolated into the droplet of Tyrode solution was then measured using a haemocytometer. Following this the sperm were then incubated at a concentration of 10^5-$10^6 ml^{-1}$ for more than three hours at 37°C, in an atmosphere of 5% CO_2 95% air. This procedure is necessary to enable the sperm to undergo capacitation, a pre-requisite for successful fertilization (Austin, 1952).

Experiments were performed in varying solutions (see 2.4) at 34-36°C on a heated microscope stage. After the egg had been impaled with a recording electrode or a cell-attached patch obtained, 50-100 μl of capacitated sperm suspension was added at the side

of the experimental chamber, to the medium bathing the egg. The final sperm concentration in the chamber was about $5000ml^{-1}$. This relatively low concentration of sperm was chosen to minimize the number of sperm reaching the egg and to maximize the interval between sperm addition and sperm attachment to the egg.

Such fertilization experiments were performed up to 18 hours after the injection of HCG.

The reason for using zona-free eggs was to avoid the delay which would otherwise be caused due to sperm passage through the zona pellucida. The time required for zona penetration in hamster eggs has been reported to be 4-22 minutes (Yang, Lin, Wang & Chang, 1972). No attempts were made to fertilize zona intact hamster or mice eggs.

Fertilization of zona-free hamster eggs usually resulted in severe polyspermy, (see Chapters 5-7 and Appendix A) as observed by previous workers (Hanada and Chang, 1972; Yanagimachi 1972b).

b) Mice

After removing the surrounding fat from the cauda epididymes, both were placed in a 1ml droplet of mouse capacitation medium (2.4g) covered with liquid paraffin. The epididymes were coarsely chopped. After 10 minutes incubation (during which time the sperm had dispersed) the tissue fragments were removed from the droplet and the sperm allowed to incubate for at least

1 more hour (at a concentration of 10^6 - 10^7 ml^{-1}) (Wolf and Stark, Inoue, 1976). This was sufficient time to allow the sperm to become capacitated.

A few heterologous fertilizations of hamster eggs with mice sperm are described in chapter 5.

2.3 Recording techniques employed in this study

A zona-free egg, was placed in a chamber mounted on an inverted microscope (Biovert, Reichert, Austria). The chamber contained a solid silver-silver chloride reference electrode in contact with the bathing solution.

During the course of this study three recording techniques were employed:-

a) Intracellular recording

b) Cell attached patch recording and

c) Whole cell recording.

a) Intracellular recording

A microelectrode (40-70 MΩ) filled with 2M potassium acetate was inserted into the egg for simultaneous potential recording and current passage. It was connected to the input of a high impedance pre-amplifier (Dagan, Model 8100 Single Electrode System, Dagan Corporation, USA). In some experiments described later on in this chapter (2.7) it was necessary to impale an egg with two independent electrodes. In these experiments one electrode was connected to the Dagan 8100 Single Electrode system and the second

electrode was connected to another high impedance pre-amplifier (Microprobe System Model M707, W.P.I. Instruments, USA). A group of experiments described in the Appendix were performed with a Dual Microprobe System Model KS700 (W.P.I. Instruments, USA). Current pulses from a Devices stimulator triggered by a Digitimer (D4030, Devices Ltd.) were passed via a bridge circuit between the barrel of the microelectrode and the bath electrode.

Cell impalement was achieved by resting the micro-electrode on the surface of the egg and increasing the negative capacity compensation applied to the microelectrode, so that it went briefly into electrical oscillation.

To monitor the membrane conductance constant current pulses (usually hyperpolarizing pulses) were applied through the intracellular electrode by means of a bridge circuit. Since the current voltage relation (I-V curve) is linear at potentials between -10 and -150 mV, changes in the amplitude of a small potential step (usually in the range 10-20mV) in response to the current pulse indicate changes of membrane conductance (Miyazaki & Igusa, 1982; Georgiou et al 1983).

b) Cell attached patch recording

Patch pipettes were made from micro-haematocrit capillary tubes (Hawksley, catalogue no.1604). They had resistances of 1-3 MΩ and internal tip diameters of

about 2 μm. The solution used to fill these patch pipettes varied with the aim of the experiment, and is therefore mentioned at each relevant point in the results section.

Some of the recordings performed during the course of this study were obtained using a laboratory made patch clamp system (based on a Teledyne Philbrick 1035 op-amp and a 1GΩ [Welwyn] feedback resistor; Martin, [1985]; Chapters 3 and 4) and the remainder (Chapters 3, 4 and 7) with the commercially available EPC-7 Patch Clamp System (List Electronic Instruments, West Germany). The laboratory made system had greater noise and limited bandwidth capabilities, when compared to the EPC-7. Unless otherwise stated, all recordings were amplified and filtered at 1000Hz, -3dB.

The patch pipette was gently lowered on to the surface of a zona-free egg and increasing suction applied to help in seal formation. A recording obtained from such an arrangement has been called a cell-attached patch recording (Hamill, Marty, Neher, Sakmann and Sigworth, 1981). Other techniques used to facilitate the formation of a high resistance seal between the egg membrane and the patch pipette were:-

(i) diluting the solution in the pipette to 90% (of the composition defined for each solution at relevant points in the text)

(ii) omitting the three sugars from the normal solution, i.e. lactate, pyruvate and glucose without any compensation for the decreased osmolarity

(iii) omitting albumin from the normal solution

(iv) pipettes used within a few hours of fabrication

(v) increasing the concentration of divalent ion in the normal solution by an equiosmolar substitution for monovalent ions, e.g. sodium chloride replaced by calcium chloride

(vi) prolonging the trypsin (for hamster eggs) or protease (for mice eggs) treatment by about 45-60 seconds (see 2.1)

(vii) polishing the pipettes. Although this is not always necessary for establishing a giga-seal it reduces the likelihood of cell damage by the pipette tip. Heat polishing was done under a microscope using a small platinum-iridium heating wire. The pipette was brought near to the heated wire until a rounding and darkening of the tip was visible.

(viii) Filtering the solution used to fill the pipette and that used to bathe the egg. The pipettes were filled by sucking a small amount of solution through the tip and then back filling.

It was usual to employ all the above techniques in any one experiment, but (i) and (ii) were not performed together in any given experiment.

Current pulses applied from a Devices stimulator triggered by a Digitimer (D4030 Devices Ltd.) were passed through the patch pipette to monitor the resistance of the seal between the patch pipette and the egg membrane.

Once a cell attached patch had been obtained, it was possible to impose voltage steps across the patch of variable magnitude and direction. It was also possible to alter the transpatch potential, by altering the pipette voltage.

c) Whole cell recording

Once a high resistance seal had been obtained with a cell attached patch, it was possible to break the patch membrane by additional suction. Electrical access to the egg interior was indicated by a sudden increase in the capacitive transients from the test pulse, and depending on the egg's input resistance, a shift in the current level. Using this mode of recording it was possible to measure membrane potentials of eggs.

Low bathing solution levels were used during whole cell recording and cell attached patch recordings because this reduced the capacitance and the coupling of the glass dielectric noise to the pipette interior.

Coating of the pipettes was not done on a regular basis, although had it been, it would have improved recordings by reducing the noise caused by the thin film of solution, which formed on the glass surface. This film generates noise which is coupled capacitively to the interior of the pipette. A hydrophobic coating (eg Sylgard) prevents the formation of such a film. On the occasions that such coatings were applied, they were made as thick as possible, and applied as close as

possible to the tip. Such precautions reduced the capacitance between the pipette interior and the bath.

Permanent experimental records were obtained as pen recorder traces on a Devices M2 recorder, as photographs of the screen of a storage oscilloscope (RM5113, Tektronix Ltd.) or stored on tape (Racal 4DS Tape recorder, Racal Recorders Ltd., U.K.). Measurements of the various parameters, of the various responses described in the text, were made from oscilloscope records and not from pen traces. Although the channel current records obtained were stored on the tape unfiltered, they have been filtered for purposes of display, and the frequency of filtering is mentioned at each relevant point (Kemo Variable Filter, Kemo Ltd., U.K.).

Initially single channel current responses were played back from the tape at one quarter of the original speed onto a pen recorder. The channel open and closed times were then analysed by hand with the aid of a Reichert Jung videoplan. Subsequently recordings on tape were analysed with the aid of a Cromemco micro-computer programmed to measure channel open and closed times. The data were played back from the tape, sampled and digitized every 200 or 400μs and continuously monitored and adjusted for slow dc drifts. An increase in current of greater than about 70% of the unit channel current was recorded as a channel opening.

After an opening, a decrease in the current to less than about 50% of the single channel current was recorded as a channel closing. The programme then determined and stored the durations of each open and closed interval in order of ocurrence. For each patch record, mean values, frequency histograms of open and closed times were displayed and plotted by the micro-computer programme.

2.4 Solutions

a) The composition of normal bathing solution

The normal solution used to bathe the eggs in most experiments, was a modified Krebs Ringer solution (BWW medium; Biggers, Whitten & Whittingham, 1971). This solution contained (mM): NaCl,120; KCl,5; $CaCl_2$,2; $MgCl_2$,1.2; Na lactate,20; Na pyruvate,1; glucose,5.6; Hepes,5; NaOH,3.2. The pH of this solution was 7.4. Bovine serum albumin was also added, to give a final concentration of $4mgml^{-1}$.

The composition of this solution was altered, according to the experiment in hand. Such alterations have been mentioned at each appropriate point in the text.

b) The composition of "high potassium" solution

Some fertilization experiments were performed in a solution which in this study has been called a high potassium solution (Chapters 5,6,7 and Appendix A). This solution was similar to normal solution except that it contained (mM): NaCl,100; KCl,25.

Fertilization experiments were performed in high potassium solutions because it is believed to resemble more closely the composition of mammalian oviducal fluid, than does the normal solution described above. Since it has been reported that mouse oviducal fluid contains 25 mM potassium (Borland, Hazra, Biggers & Lechene, 1977; normal solution contains 5mM potassium).

c) The composition of "low sodium" solution

This solution was similar to high potassium solution except that it did not contain sodium chloride. The latter had been replaced by either choline chloride or lithium chloride on an equimolar basis. Experiments in such solutions are described in Chapter 5.

d) Calcium free solutions containing other multivalent ions

Some fertilization experiments were performed in normal or high potassium solutions from which calcium chloride was omitted. These solutions have been called calcium free solutions. Calcium free solutions were supplemented with one of four multivalent cations, i.e. Barium, Lanthanum, Magnesium and Strontium. Such experiments have been described in Chapter 6. The concentrations of each of these "substitution" ions, is noted in the text of Chapter 6.

In some experiments the sodium chloride in normal solution was partially or completely replaced by one of the three divalent ions mentioned above, on an

equiosmolar basis (Chapters 3-7 and Appendix A).

e) Abbreviations used throughout the text to describe the composition of bathing solutions

The composition of the different bathing solutions was designated by the chemical symbol of the divalent ion used in that solution, followed by its concentration in millimoles per litre. The concentration of potassium in that solution was similarily indicated. Hence normal solution is designated as Ca2K5, high potassium solution as Ca2K25 and a normal solution in which calcium is replaced by 5 mM strontium chloride as Sr5K5.

f) The composition of hamster capacitation medium

The solution in which hamster sperm were isolated and capacitated was a modified Tyrode solution containing (mM): NaCl,125; KCl,2.7; $CaCl_2$,1.8; NaH_2PO_4,0.4; $MgCl_2$,0.5; Taurine,0.5; $NaHCO_3$,11.9; Na pyruvate,0.1; sodium lactate,9.0; L adrenaline (Sigma),0.05; glucose, 4.5; Bovine serum albumin (Fraction V, Sigma),30mgml^{-1}. (equilibrated with 5% CO_2 95% air; pH 7.4).

g) The composition of mouse capacitation medium

The solution in which mouse sperm were isolated and capacitated was a modified Tyrode solution containing (mM): NaCl,97.9; KCl,1.4; $CaCl_2$,1.8; Na_2HPO_4,0.3; $MgCl_2$,0.5; $NaHCO_3$,25; Na pyruvate,0.5; Na lactate,25; glucose,5.6; Bovine serum albumin,15mgml^{-1} (Fraction V, Sigma) (equilibrated with 5% CO_2 95% air; pH approximately 7.5-7.7). Both the mouse and hamster

capacitation media were made up with double glass distilled water.

2.5 Visual observation of fusion

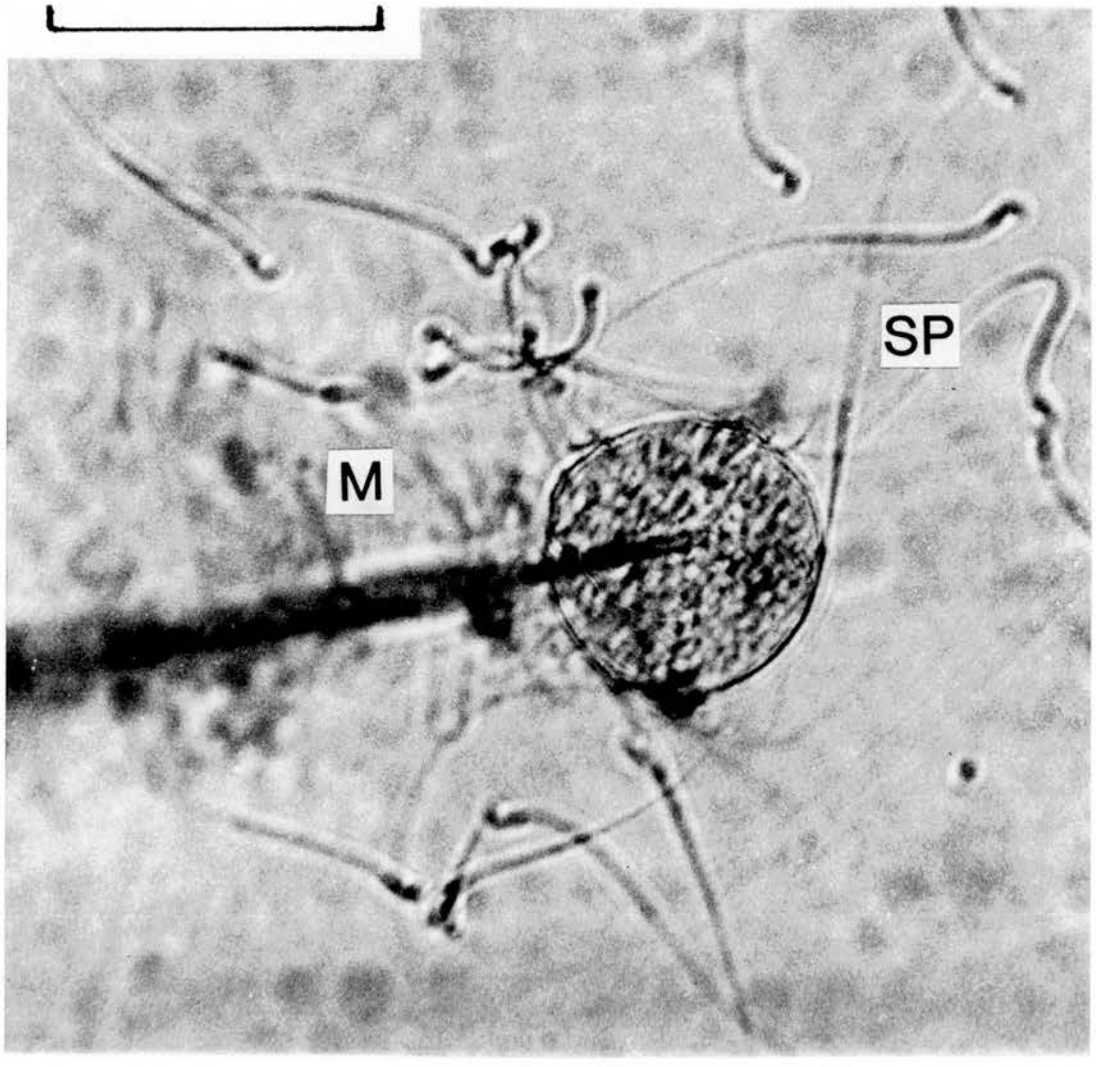
The instant of fertilization (fusion of sperm with the egg) is reported as being the instant when the sperm tail straightens and becomes immotile (Yanagimachi, 1978a). During the course of these experiments it was found that the fertilizing sperms did not always lose their motility suddenly and indeed in most cases, the motility dropped over a period of up to 30 seconds. Therefore electrical responses recorded in this study have been described as being associated with sperm fusion. In some experiments immotile sperm could appear motile, because of movement caused by adjacent motile sperm, either attached to the egg or swimming freely in the surrounding bathing medium.

Throughout all the figures in this study, arrows have been used to denote sperm fusions. These were the best estimates possible for the "instants of fusion", but for the reasons mentioned above, should be treated with a little caution.

Figure 2.1 shows a microscope picture of an impaled egg to which is attached a straight and immotile sperm. This sperm had fused with the egg and caused fertilization as confirmed by subsequent histological examination.

Figure 2.1

A photograph of a zona-free hamster egg impaled with a recording microelectrode (M). Several sperm were in contact with the egg but only one was both immotile and straight (SP). This photograph was taken down the eye piece of the inverted microscope. The scale bar is 100μm.


SP
M

2.6 Histological confirmation of fertilization

It was a necessary part of each experiment to find out histologically, whether or not the egg (from which a recording had been obtained) was fertilized (Yamashita, 1982). This was done by removing the recording microelectrode and/or the patch pipette (the latter removed by gradually releasing suction, and lifting the pipette clear away from the egg) from the egg, at the end of the experiment. The egg was transferred from the chamber to a small drop of the sperm free bathing medium (i.e. whichever solution the egg was bathed in during the course of the experiment). The only exception to this rule was one group of experiments in which fertilization was attempted in a calcium free normal solution containing 1mM lanthanum (see Chapter 6). The eggs in this group of experiments, were transferred at the end of the recording to a small drop of normal bathing solution. The drop of sperm free bathing solution containing the egg was covered with liquid paraffin, and allowed to incubate for up to four hours at 37^{o}C in an atmosphere of 5% CO_2 95% air. After this period of incubation the egg was fixed in neutral buffered formalin (10% formaldehyde), and transferred as a small drop to a microscope slide. The egg was compressed with a cover slip mounted on four small supports of a 20:1 vaseline : liquid paraffin mixture. Ethanol was introduced into the gap between the slide and the cover slip, and drawn through using a paper tissue as a wick. The perfusion

Figure 2.2

A,B and C are phase contrast micrographs of a fertilized zona-free egg taken at three different planes in the egg. A shows a pronucleus (P1) and an enlarged sperm head (SH). B shows two further pronuclei (P2 and P3), and C shows one more pronucleus (P4) and a polar body (PB). So in this particular egg there were four pronuclei and one enlarged sperm head, indicating that the egg had been fertilized by four sperm. This egg was fertilized in normal solution. D is a light micrograph of another egg (fertilized in high potassium solution, Ca2K25) containing an enlarged sperm head (SH). E is a phase contrast micrograph of an egg which was fertilized in high potassium solution, Ca2K25. The second polar body is visible (PB), as well as other sperm which were in contact with the egg membrane but had not fused with it. The outline of these sperm heads is distinct and they are smaller in size than the enlarged sperm heads seen in A and D. Usually the diameter of an egg is about 80μm. Each egg shown in A-E has been compressed under a coverslip to a final diameter of about 200μm.

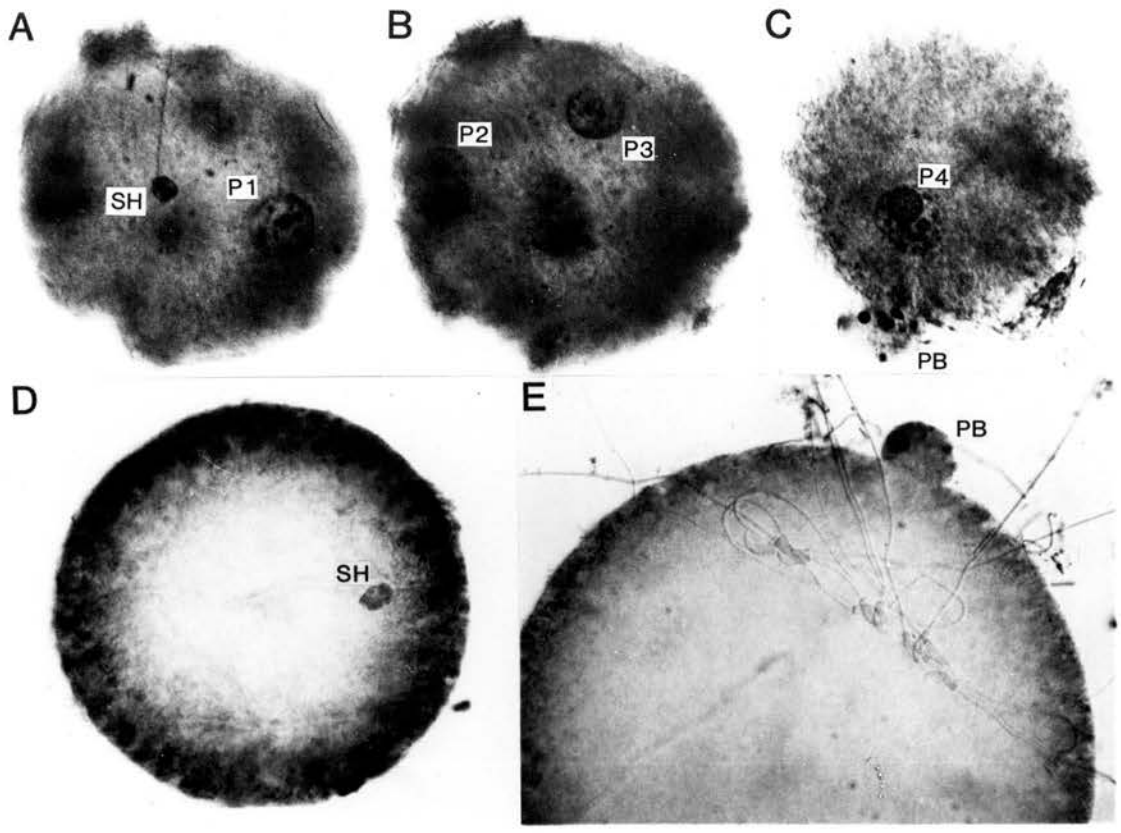
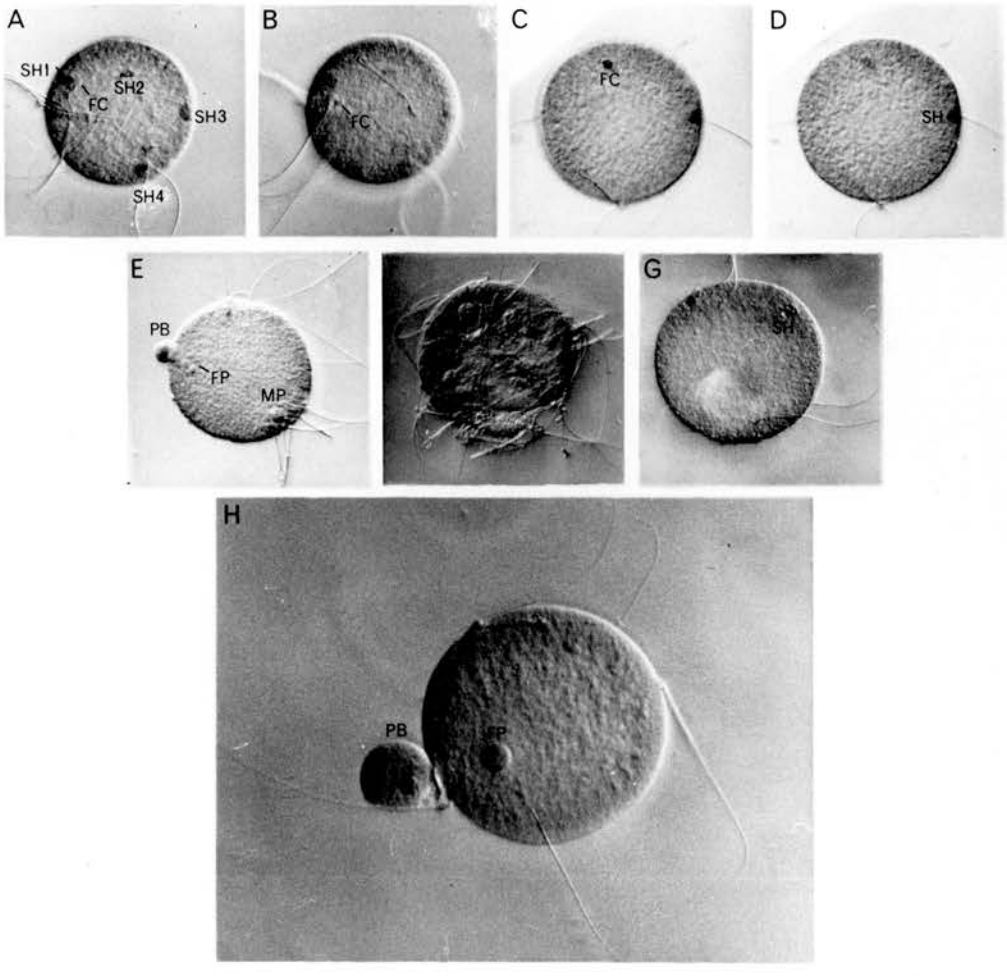

A
SH
P1
B
P2
P3
C
P4
PB
D
SH
E
PB

Figure 2.3

A-H are micrographs of zona-free hamster eggs (compressed under a coverslip to a diameter of 200μm) observed with Nomarski optics. Those shown in A-E were from eggs fertilized in normal solution containing 4mM calcium (Ca4K5). A shows an egg containing four enlarged sperm heads (SH1 to SH4). Also visible are the female chromosomes (FC). B is a micrograph of the same egg as in A, but at a different plane showing two sperm which have not participated in fertilization. The egg shown in A and B was fertilized by four sperm. C and D are micrographs from an egg fertilized by only one sperm, showing respectively, the female chromosomes (FC) and the enlarged sperm head (SH). E shows a polar body (PB), a male pronucleus (MP) and a female pronucleus (FP) (the latter is near to the polar body, but slightly out of focus). F shows an egg (fertilized in high potassium solution, Ca2K25) containing five pronuclei (P1-P5), therefore indicating that it had been fertilized by four sperm. It is not possible in this example to indicate which of these pronuclei is a female pronucleus. G illustrates an egg fertilized by one sperm in Sr5K25. The enlarged sperm head (SH) is visible, and also the associated tail, lying close to the circumference of the egg. H. Female pronucleus (FP) and the second polar body in a spontaneously activated egg.


A
SH1
SH2
FC
SH3
SH4
B
FC
C
FC
D
SH
E
PB
FP
MP
G
SH
H
PB
FP

was repeated using 0.25% lacmoid in 45% acetic acid, (it was not necessary to wait between successive steps during the fixation procedure) for visualization of egg chromosomes, sperm heads or pronuclei.

Eggs were subsequently observed under light microscopy, phase contrast microscopy or using Nomarski optics at 400X. Eggs were considered to be fertilized if they had resumed meiosis II and possessed a decondensing sperm head or a male pronucleus, and an associated polar body. Decondensing sperm were identifiable by their larger size and indistinct outline.

Figures 2.2 and 2.3 illustrate examples of decondensing (enlarged) sperm heads (2.2A, 2.2D, 2.3A, 2.3D and 2.3G), pronuclei (2.2A, 2.2B, 2.2C, 2.3E and 2.3F) polar bodies (2.2C, 2.2E, 2.3E and 2.3H) and also sperm which have not participated in fertilization (2.2E, 2.3B and 2.3G).

Since in mice eggs, a maximum number of decondensed sperm per egg is attained by 60 minutes post-insemination, in zona-free eggs inseminated under similar conditions (Yu & Wolf, 1981), it was concluded that non-decondensing sperm associated with eggs fixed later than 60 minutes post-insemination are non-fertilizing sperm.

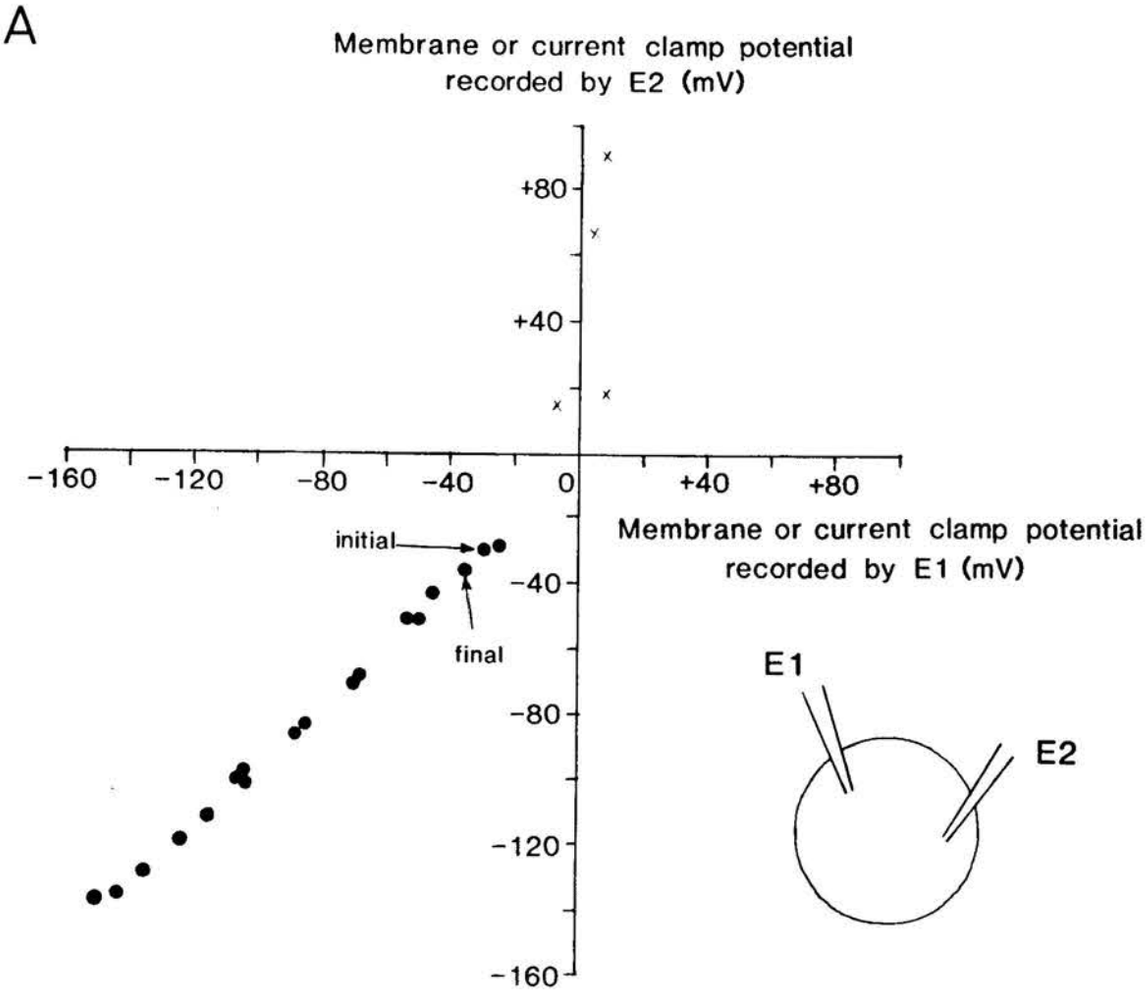
2.7 Control clamp experiments

During the course of this study some experiments

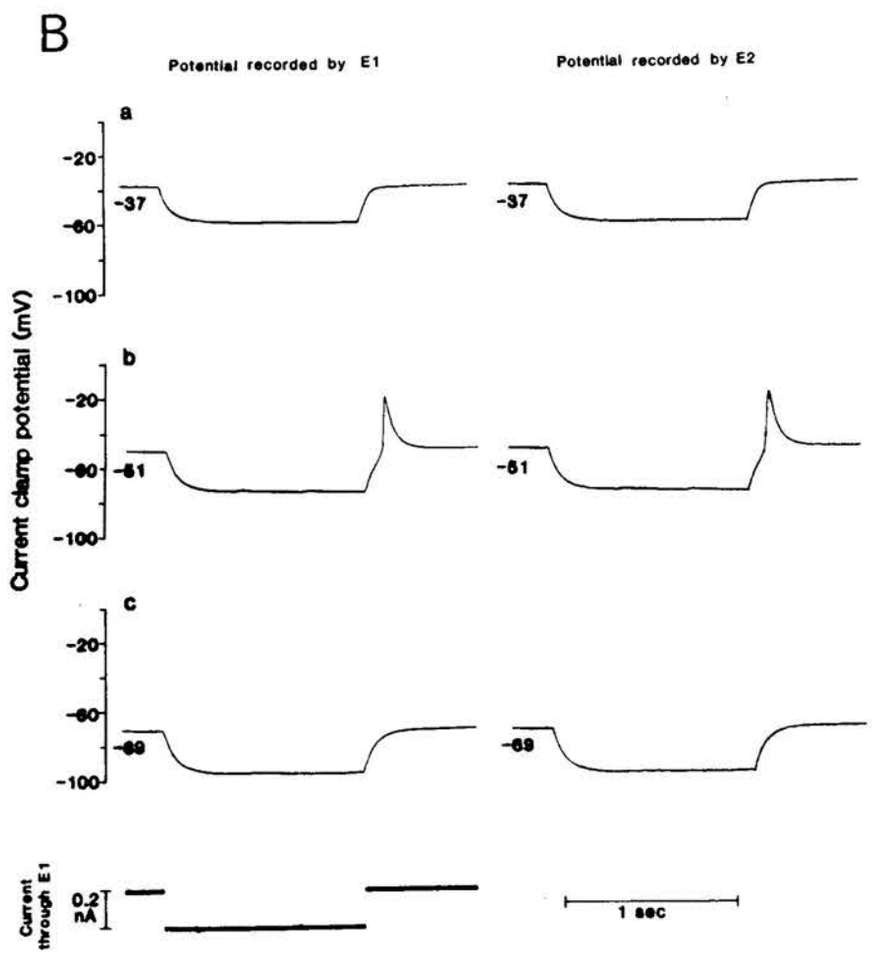
Figure 2.4

A.Relationship between the membrane potential recorded by two independent intracellular microelectrodes (E1 and E2) inserted into the same egg. Steady hyperpolarizing or depolarizing current was passed through E1 to clamp the egg at various potentials. The point marked "initial" corresponded to the potentials recorded by E1 and E2, 6.2mins after inserting the second electrode. The point marked "final" corresponded to the potentials recorded by E1 and E2, when all steady current had been removed, after having hyperpolarized the egg as far as -140 mV, approximately. The four points marked by crosses,indicate that when constant depolarizing current was passed through E1, the potentials recorded by both electrodes were no longer similar. B.Oscilloscope pictures of responses recorded simultaneously by E1 and E2, as a result of passing hyperpolarizing pulses through E1 (0.2nA, 0.2Hz, 1.2sec). C Oscilloscope pictures of responses recorded by E1 and E2 as a result of passing depolarizing pulses through E1. The pulses in a and b were 0.2nA, 0.2Hz and 1.2sec, and those in c were 0.48nA, 0.2Hz and 1.2sec. The numbers at the bottom left hand corner of each oscilloscope trace, are the current clamp potentials recorded by each electrode. A-C were obtained in the same egg, bathed in normal solution containing 4mM calcium chloride (Ca4K5).

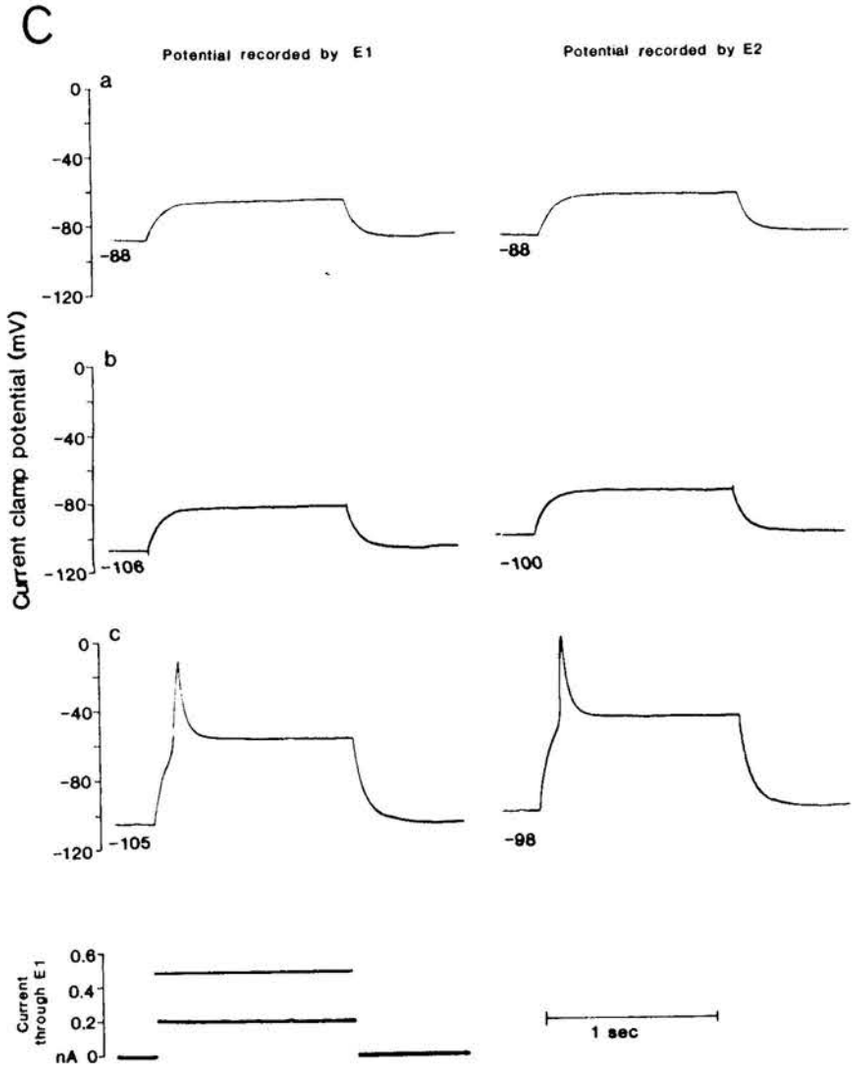
A


Membrane or current clamp potential recorded by E2 (mV)
+80
+40
-40
-80
-120
-160
-160
-120
-80
-40
0
+40
+80
initial
final
Membrane or current clamp potential recorded by E1 (mV)
E1
E2

B


Potential recorded by E1
Potential recorded by E2
Current clamp potential (mV)
a
b
c
-20
-60
-100
-37
-51
-69
Current through E1
0.2 nA
1 sec

C


Potential recorded by E1
Potential recorded by E2
Current clamp potential (mV)
a
b
c
0
-40
-80
-120
-88
-106
-105
-100
-98
Current through E1
0.6
0.4
0.2
nA 0
1 sec

were performed under current clamp conditions (some experiments in Chapter 4, all but three fertilizations scored in Chapter 5, all but one experiment in Chapter 6, some of the experiments in Chapter 7 and Appendix A). It was therefore necessary to ensure that the microelectrode under current clamp conditions was monitoring the true membrane potential, and not an artifact caused by the passage of constant current through the recording electrode (which could be quite large if the input resistance of the egg was low relative to the microelectrode resistance). The error in the measured membrane potential being appreciable with high resistance electrodes, since it is equivalent to IR, where I is the constant current passed through the electrode and R is the resistance of the electrode.

Throughout this study the term membrane potential is used to refer to situations in which no steady current is being passed through the recording electrode, i.e. zero current clamp situations. Whereas the term "current clamp potential" is used to describe eggs which are hyperpolarized or depolarized by the passage of steady current through the electrode.

Figure 2.4A shows how the potentials recorded by two electrodes inserted into one egg compared with each other, at different current clamp potentials. Another example of such an experiment is illustrated by Fig.2.5A. The electrode through which constant current is passed to current clamp the egg at various potentials, and to pass hyperpolarizing or depolarizing

current pulses across the egg membrane has been designated E1 (connected to the Dagan 8100 Single Electrode System). The other electrode is designated E2 (connected to the Microprobe System, Model M707).

The results illustrated by Fig. 2.4A were obtained from an egg impaled with two electrodes with resistances of 50(E1) and 60MΩ(E2). When a rectangular hyperpolarizing current pulse was passed through the recording electrode (E1), an electrotonic potential was recorded. The input resistance of the egg was calculated by dividing the amplitude of the electrotonic potential by the size of the current pulse. After inserting the second electrode (E2), a period of 6.2 minutes was allowed to elapse before current clamping the egg. During this period the membrane potential and input resistance (as monitored by both electrodes) gradually increased, presumably because the plasma membrane progressively sealed to both electrodes (see Chapter 4). The membrane potential and input resistance stabilized at -29mV and 94MΩ (as monitored by E1, at which point E2 recorded -29mV and 98MΩ). This value for the membrane potential (under zero current clamp) of -29mV, as recorded by both electrode E1 and E2, has been marked as "initial" in Fig. 2.4A. The egg was then current clamped at various potentials by passing steady hyperpolarizing currents through E1, and the current clamp potentials monitored by both E1 and E2 were noted. In this egg,

with the above mentioned electrodes, the potential correlation between the two electrodes was 0.999 (n=17) in the range -29mV to about -140mV.

After removing all steady hyperpolarizing current, the potential then monitored by both electrodes was -36mV and has been marked "final" in Fig.2.4A. At this point constant depolarizing current was passed through E1, and the potentials monitored by both electrodes recorded. It was found that for large depolarizing currents passed through E1, the correlation became very poor - as denoted by the four points which have been marked by crosses in Fig. 2.4A. When a very small depolarizing current was passed through E1 the current clamp potential monitored by E1 was -25 mV and that by E2 was -28mV. This point and the four which are marked by crosses in Fig. 2.4A were ignored in the linear regression analysis.

At the end of this experiment, the membrane potential and input resistance monitored by E1 was -32mV and 60MΩ, and by E2 was -31mV and 70MΩ (these values of the measured membrane potential are corrected for tip potentials of -1 and -2 mV, in E1 and E2 respectively).

Figure 2.4B shows oscilloscope pictures of responses recorded by E1 and E2, during the passage of hyperpolarizing pulses through E1 whilst current clamping the egg at three different potential levels (-37mV, -51mV and -69mV as recorded by both electrodes). The hyperpolarizing pulse was identical in

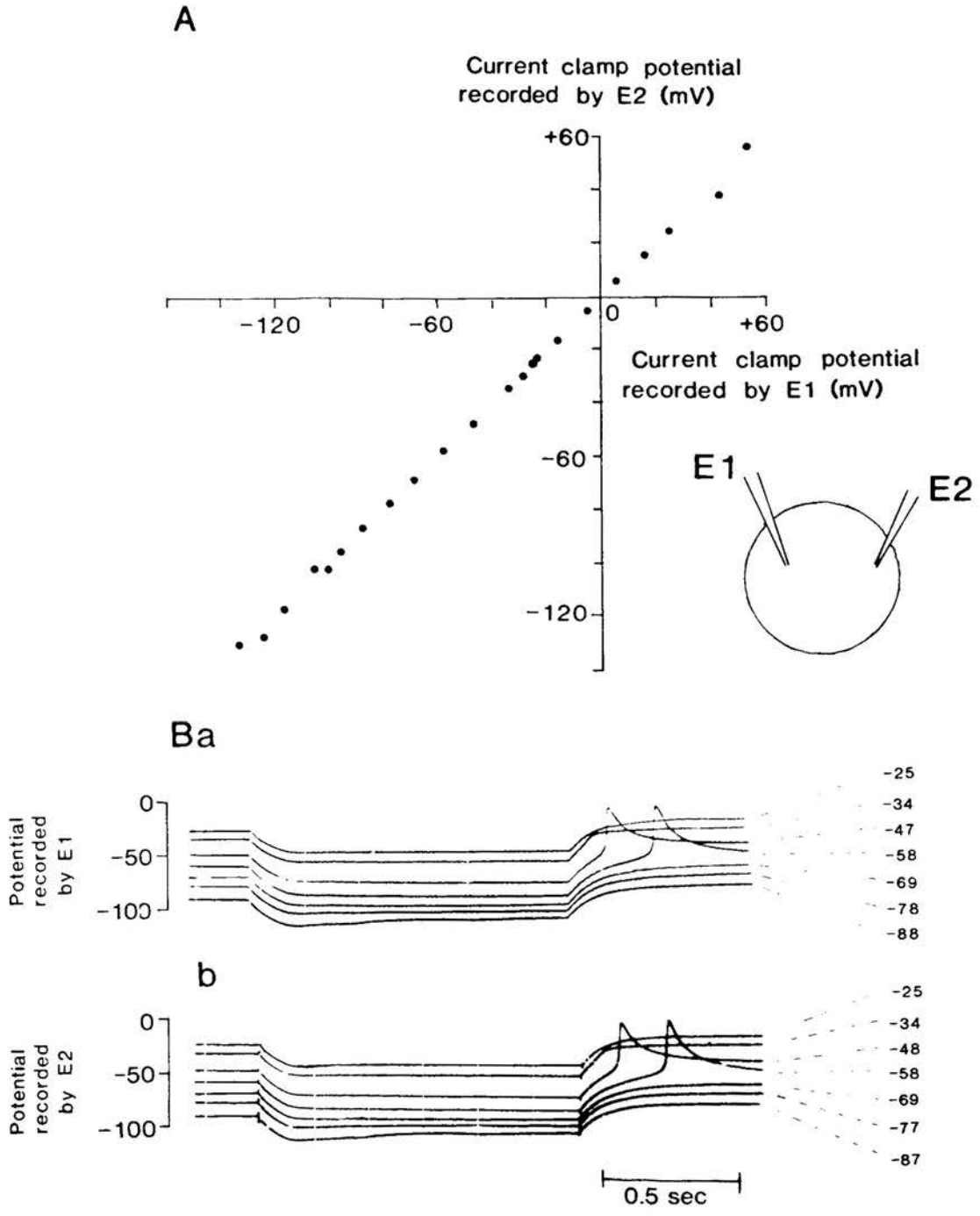
all three situations illustrated in Fig. 2.4B (0.2nA, 1.2 sec, 0.2Hz). The response recorded by both electrodes was identical at the three current clamp potential levels shown.

Figure 2.4Bb shows that anode break responses occur if the hyperpolarizing pulse is of sufficient magnitude (dependent on the input resistance of each egg, and the membrane or current clamp potential of the egg), to hyperpolarize the egg below the threshold for such a response. Hence at the end of the pulse, as the membrane potential returns to it s value prior to the pulse, it passes through the threshold for such a response and therefore elicits an anode break spike (Fig. 2.4Bb). When the current clamp potential was -37mV (Fig. 2.4Ba), the pulse was not sufficiently large to hyperpolarize the membrane to below threshold for eliciting such an anode break response (the threshold from Fig. 2.4Bb is about -55mV). Hence in that situation, if the size of the pulse had been increased, anode break excitation would have been observed. Analysis of such anode break responses in mouse and hamster eggs, has indicated that they are due to the opening of calcium channels (Okamoto, Takahashi and Yamashita, 1977; Miyazaki and Igusa, 1982; Eusebi, Colonna and Mangia, 1983; Yoshida, 1983).

If the membrane or current clamp potential of the egg is more hyperpolarized than the threshold, (as in Fig. 2.4Bc, where the current clamp potential was

Figure 2.5

A. Relationship between the potentials monitored by two independent intracellular electrodes in an egg, bathed in Ca4K5. The egg was clamped at various potentials by passing depolarizing or hyperpolarizing currents through one of the electrodes (E1). B.a shows seven oscilloscope responses recorded by E1 and b shows seven oscilloscope responses recorded by E2 simultaneously, as a result of passing hyperpolarizing pulses through E1 (0.2nA, 0.2Hz, 1.2sec). Each of the responses shown corresponds to a different current clamp potential. The current clamp potential was altered by varying the steady current passed through E1. The figures shown on the right of each series of oscilloscope pictures corresponds to the current clamp potential recorded by each respective electrode. Passive electrotonic responses were observed at current clamp potentials of -25,-69,-78 and -88mV, whereas anode break responses were observed at current clamp potentials of -34,-47 and -58mV (all these current clamp potential values are those recorded by E1). The results shown in A and B were obtained in the same egg.


A
Current clamp potential
recorded by E2 (mV)
+60
-120
-60
0
+60
Current clamp potential
recorded by E1 (mV)
-60
-120
E1
E2
Ba
Potential
recorded
by E1
0
-50
-100
-25
-34
-47
-58
-69
-78
-88
b
Potential
recorded
by E2
0
-50
-100
-25
-34
-48
-58
-69
-77
-87
0.5 sec

-69mV), then hyperpolarizing pulses result in passive electrotonic responses (Fig. 2.4Bc). Such responses were used throughout this study to monitor the input resistances of the eggs.

When the egg was current clamped at potentials of -88mV and -106mV (as monitored by E1), the passage of a depolarizing pulse (0.2nA, 1.2sec, 0.2Hz) resulted in electrotonic responses as shown in Fig. 2.4Ca and 2.4Cb respectively. When the size of the depolarizing pulse was increased to 0.48nA, then an action potential was observed (Fig. 2.4Cc). Such an action potential in this study has been called an electrically evoked action potential. The threshold for the electrically evoked action potential corresponds to that of the anode break response in any given egg. This is because the electrically evoked action potential is due to the influx of calcium through the same channels which open during an anode break response (Georgiou et al , 1984).

All the responses described above (as a result of hyperpolarizing or depolarizing pulses), when monitored by the two electrodes (E1 and E2) simultaneously, were in fact identical up to current clamp potentials of -88mV. There are two major conclusions to be drawn from such experiments. Firstly, the eggs can be current clamped reliably with the Dagan Single Electrode System, and secondly, that the input resistance of a egg can be measured reliably with a single electrode.

In a similar experiment illustrated in Fig. 2.5A, the resistance of the current passing electrode (E1)

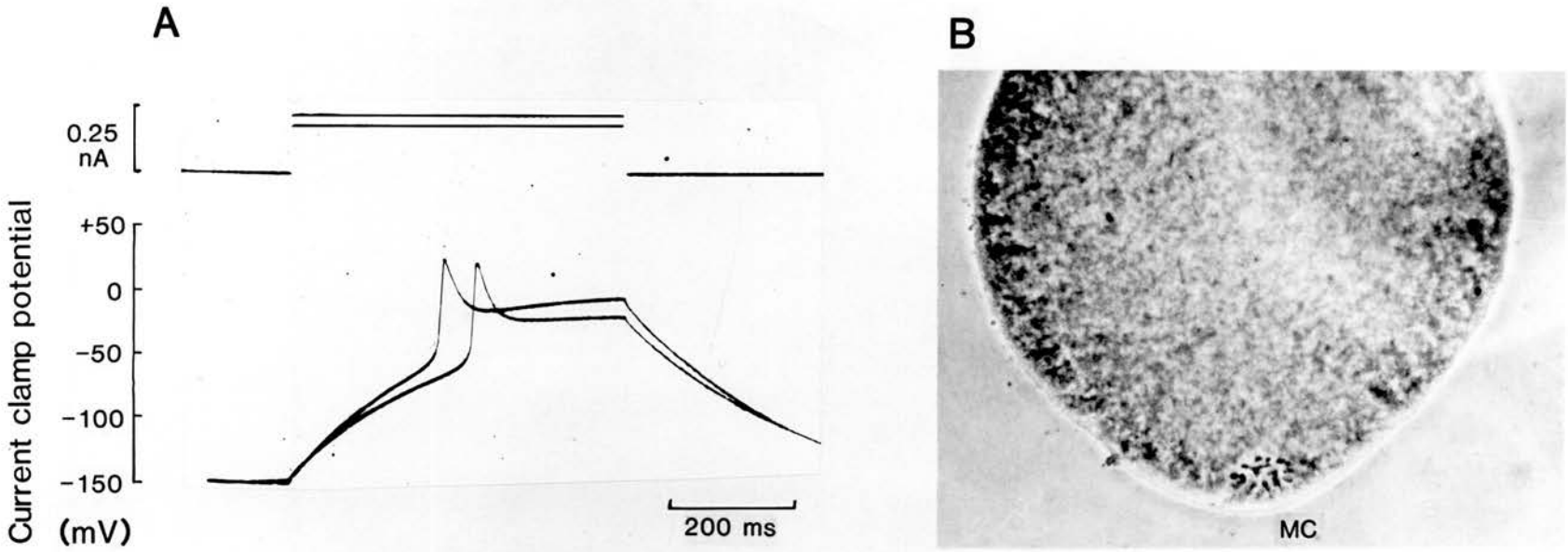
was lower (26MΩ) than that in the above mentioned experiment, and that of the secondary recording electrode (E2) was 58 MΩ. (The resistance of this second recording electrode was comparable in this experiment to that in the experiment illustrated by Fig. 2.4A.) In this egg the potential correlation between the two electrodes was almost unity (correlation coefficient = 0.999, n=22), for current clamp potentials in the range -134mV to +53mV. In this egg, the linear correlation, even for depolarizing currents was presumably because of the lower resistance of E1. In this example, after allowing adequate sealing of the second electrode, the membrane potential and input resistance (under zero current clamp), as monitored by both electrodes was -25mV and 92MΩ. At the end of the experiment, E1 registered -24mV and 80MΩ, whereas E2 registered -26mV and 80MΩ. The tip potential for both electrodes was zero.

2.8 Do electrically evoked action potentials activate zona-free hamster eggs?

As part of the experimental protocol for fertilization experiments (performed using intracellular electrodes), the majority of eggs underwent anode break responses during the period between impalement and insemination. Of the 27 fertilization experiments attempted on zona-free hamster eggs in normal bathing solution (Ca2K5), 19 were successful. In all eight failures anode break

Figure 2.6

Nine action potentials were electrically evoked by passing depolarizing current pulses, through the recording microelectrode, in a zona-free egg. The current clamp potential of this egg was about -150mV. Two action potentials are shown in the oscilloscope picture A. The upper trace shows the depolarizing current pulses used to evoke the action potentials shown in the lower trace, both of which are overshooting. After removing the electrode the egg was incubated for four hours in sperm free medium, then fixed in neutral buffered formalin (10% formaldehyde) and stained with 0.25% lacmoid in 45% acetic acid. B. Phase contrast micrograph of the egg after this staining procedure: it has been compressed to a diameter of about 200μm. The female chromosomes (MC) remained at Metaphase II of the second meiotic division.

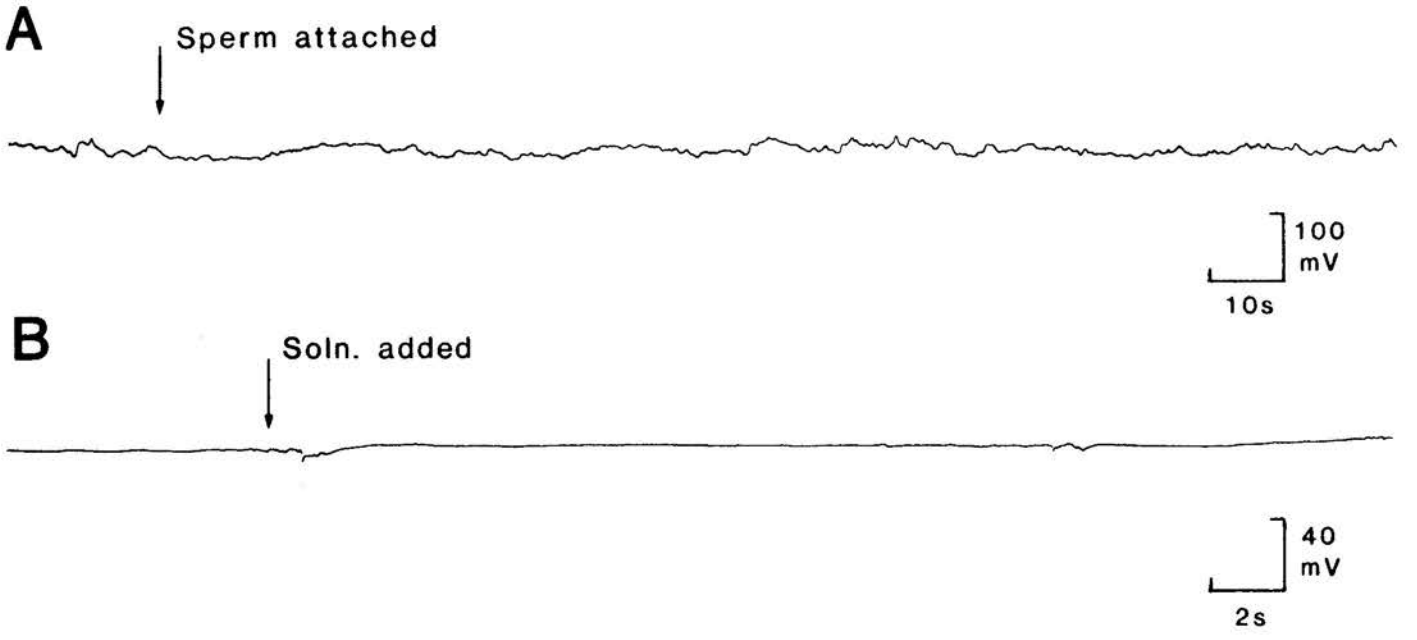

A
0.25
nA
+50
0
−50
−100
−150
Current clamp potential
(mV)
200 ms
B
MC

responses were elicited during the period between impalement and insemination. Since none of these failures showed signs of parthenogenetic activation, it can be concluded that the calcium influx during the anode break responses was insufficient to activate the eggs. Furthermore, in three out of the eight failures electrically evoked action potentials were elicited (for measuring various parameters - see Chapter 5) prior to insemination. In several experiments on eggs current clamped at potentials below threshold, action potentials were evoked by depolarizing current pulses. The results of such an experiment are shown in Fig. 2.6. Nine action potentials were elicited in this egg, two being illustrated in Fig. 2.6A. Histological examination revealed that the egg chromosomes had remained at Metaphase II of the second meiotic division (Fig. 2.6B), i.e. no female pronucleus had been formed nor had the second polar body been released.

Similarly in the group of experiments described in Appendix A, of the 97 eggs bathed in normal solution 53 did not become fertilized in the presence of sperm (all eggs underwent anode break responses during the period between impalement and insemination). None of these failures showed signs of parthenogenetic activation. Even though in some of these failures, electrical action potentials had been evoked.

Figure 2.7

Pen traces of the membrane potentials of two eggs studied in control experiments. In A a sperm attached itself to the egg and continued to beat its tail vigorously. In B a small volume of modified Tyrode solution was added to the solution bathing another egg. The current clamp potential of the egg in A was -120mV and that in B was -90mV.


A
Sperm attached
100
mV
10s
B
Soln. added
40
mV
2s

2.9 Controls for the fertilization experiments

Several kinds of control experiments were done on unfertilized eggs to determine whether (a) the contact of motile sperm without fusion with the egg, (b) the composition of medium in which the sperm are suspended and (c) the mechanical displacement of an egg might alter the egg's membrane potential.

Figure 2.7A and B respectively, show examples of intracellular recordings from eggs in type "a" and "b" control experiments. In Fig. 2.7A the attachment of motile sperm (arrow) to the egg, had no influence on the potential. Histological examination confirmed that the egg was not fertilized. When a small volume (50 μl) of modified Tyrode solution was added to the chamber (arrow in Fig. 2.7B), in a manner similar to that of sperm addition, the potential of another egg did not change significantly, nor was the egg activated. Control experiments of type "c", where large volumes (500μl) of normal solution were added to the chamber, or eggs were prodded by coarse microelectrodes, failed to reveal a mechano-sensitive response of eggs, either electrically or histologically.

Of the eight failed fertilizations in Ca2K5, none showed electrical responses (like those described in Chapters 5-7 and Appendix A), after the addition of sperm to the medium bathing the egg.

CHAPTER	TYPE OF EXPERIMENT		SPECIES OF EGG	
			HAMSTER	MOUSE
Chapter 3	The use of cell attached patches to study:-	Calcium channels	√	√
		Calcium activated potassium channels	√	√
Chapter 4	Whole cell recording to measure membrane potentials (with patch pipettes).		√	√
Chapters 5 & 6	Fertilization monitored using intracellular electrodes:-	Using hamster sperm	√	X
		Using mice sperm	√	X
Chapter 7	Fertilization monitored using cell attached patches:-	Using hamster sperm	√	X
		Using mice sperm	X	X

TABLE 2.1

Table 2.1

This is a summary of the types of experiments described in the "results chapters" (i.e. 3-7) of this study. Not every type of experiment was performed on both species of egg (crosses).

2.10 Summary (Table 2.1)

Table 2.1 shows the different types of experiments performed on the two species of eggs examined in this study.

The first part of the results section, (i.e. Chapters 3-4) describes results obtained from unfertilized eggs. Chapter 3 describes a preliminary investigation, of the types of channels present on mouse and hamster egg membranes. In Chapter 4 the problems involved in measuring the membrane potential in this preparation are discussed, and results obtained from whole cell recording measurements are described. Also in Chapter 4, channel openings in response to depolarizing or hyperpolarizing pulses are illustrated.

The remainder of the results section is devoted to fertilization experiments (Chapters 5-7). Both Chapters 5 and 6 relate to fertilization experiments employing intracellular electrodes. Chapter 5 analyses experiments in which the egg was bathed in normal or high potassium solution or either of these solutions with elevated calcium (by equiosmolar replacement of sodium chloride). Chapter 6 looks at experiments similar to those in Chapter 5, but the calcium is replaced by other di - or trivalent ions (i.e. Barium, Lanthanum, Magnesium and Strontium). Then finally in Chapter 7, the extracellular recordings made during fertilization (i.e. cell attached patch recordings) are discussed.

In Appendix A are described results of experiments, similar to those illustrated in Chapter 5. The reason that this group of results has been treated in the Appendix, is that they are believed to be qualitatively correct but quantitatively in error (because of instrument malfunction).

CHAPTER 3 A PRELIMINARY INVESTIGATION OF THE TYPES OF CHANNELS PRESENT ON THE MOUSE AND HAMSTER EGG MEMBRANE

In this chapter are described, some of the channel current recording experiments performed during this study. The results (all of which are cell attached patch recordings) give a preliminary indication of the types of channels present on the egg membrane. Most of the experiments were performed on zona-free mouse eggs, because in general, it was observed that the resistance of the seal between the patch pipette and the egg membrane was greater than in similar experiments with zona-free hamster eggs. This may be due to the fact that there are more areas devoid of microvilli, and there are fewer microvilli per unit area, in mouse egg membranes compared to hamster egg membranes. It has been reported in mouse eggs that numerous microvilli are associated with cortical granules beneath the plasma membrane, whereas areas devoid of granules tend to be smooth (Nicosia, Wolf & Inoue, 1977). Since cortical granules are absent in much of the mouse egg hemisphere homolateral to the meiotic spindle, it is assumed that there are fewer or no microvilli in this half of the egg (Nicosia et al, 1977). Also in other species of mammalian eggs, it is reported that the freshly ovulated oocytes exhibit numerous microvilli over the entire surface, except at the region superficial to the meiotic apparatus, where the plasma membrane is smooth (Gulyas, 1976; Nicosia et al., 1977).

3.1 Methods

Four types of solution were used to fill the patch pipettes. They were:-

a) Cal0K5 (90%). This was normal solution (composition given in 2.4a) containing 10mM calcium chloride, which had been diluted to 90% with glass distilled water. Possible currents with this solution were calcium, chloride, potassium or sodium.

b) Sr80K5. The composition of this solution was mM: $SrCl_2$,80; KCl,5; $CaCl_2$,1; KOH,2.5 and HEPES,5. Currents which could be recorded with this solution were calcium, strontium (via the calcium channels), chloride or potassium. No sodium was present in this solution.

c) Sr60/TEA/CsCl. The composition of this solution was mM: $SrCl_2$,60; $CsCl_2$,15; $CaCl_2$,1; TEA-Cl,20; KOH,2.5 and HEPES,5. The purpose of adding caesium chloride and TEA-Cl (Tetra-ethyl ammonium chloride), was to try and abolish any potassium currents. Theoretically calcium, strontium, and chloride currents could still have been observed.

d) K155 + EGTA. The composition of this solution was mM: KCl,155; $MgCl_2$,2; $CaCl_2$,0.01; EGTA,1; KOH,2.5 and HEPES,5. This solution contained 10^{-9}M free calcium, assuming that calcium-EGTA has a dissociation constant of 10^{-7}, at pH 7.2 (Fenwick,Marty & Neher, 1982a). In such a solution calcium currents and sodium currents should not be observed, and the reversal potential for

potassium should be 0mV (assuming that the concentration of potassium in the cytosol of eggs is 155mM). Chloride currents may still be observed with this solution in the pipette.

High concentrations of divalent ion (e.g. 11.2mM in Ca10K5, 81mM in Sr80K5 and 61mM in Sr60/TEA/CsCl) were used in the pipette filling solutions, to increase the amplitude of currents due to divalent ions, and to promote the sealing between the patch pipette and the egg membrane.

During these experiments the eggs were bathed in four types of solution:-

a) Ca4K5 - normal solution containing 4mM calcium chloride.

b) Ca10K5 - normal solution containing 10mM calcium chloride,

c) Ca4K5 + La2 - normal solution containing 4mM calcium chloride and 2mM lanthanum nitrate and

d) Sr80K5 - composition given above.

Experiments indicated that a concentration of divalent ion above that in normal solution, and or the presence of lanthanum nitrate promoted the sealing between the patch pipette and the egg membrane.

The temperature of the bathing solution in all the experiments described in this chapter was 34^{o}C unless otherwise stated.

In all the figures an outward current is indicated by an upward deflection. 1, 2, 3, 4 or 5 channels simultaneously open, are indicated by O_1, O_2, O_3, O_4 or

O_5 respectively. C indicates that all the channels are closed

Since the exact resting potential of any given egg was unknown, transpatch potentials have been indicated by depolarizations or hyperpolarizations of the patch, by the imposed pipette potential. For example, a pipette potential of -40mV, served to depolarize the patch by 40mV and the transpatch potential has therefore been denoted as D40 (i.e. in this situation the potential difference across the patch was 40mV more positive than the resting potential of the egg - see 3.8). Similarly, H30 denotes a pipette potential of +30mV and a transpatch potential which is 30mV more hyperpolarized than the resting potential of the egg. When no pipette potential is imposed across the patch, the transpatch potential is then equivalent to the resting potential of the egg, indicated by "0" or "RP".

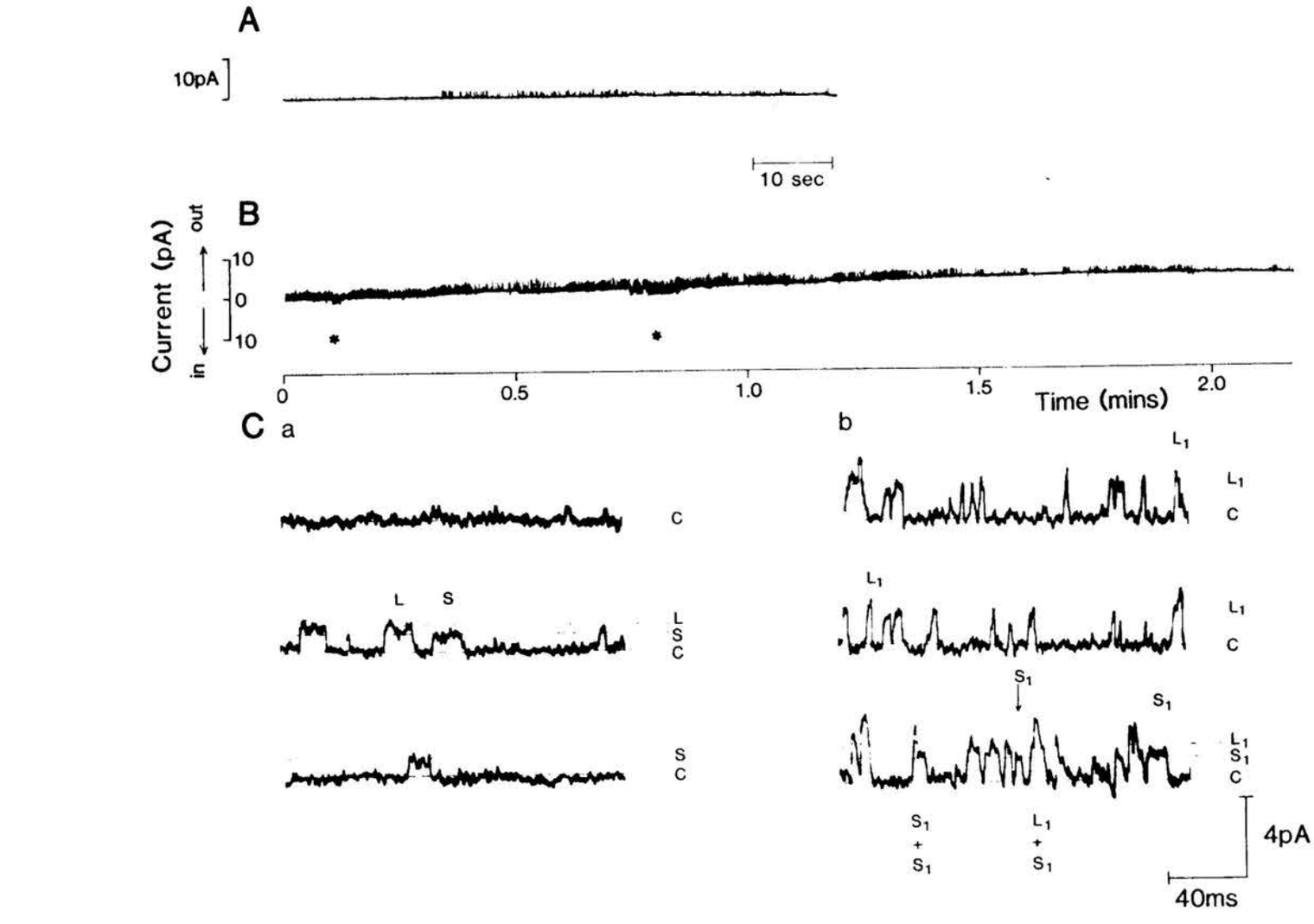
All experiments discussed in this chapter were performed with the laboratory made patch clamp apparatus, except those in Section 3.7 (in the latter the recording was done with the commercially available "List" apparatus - see Chapter 2.3b).

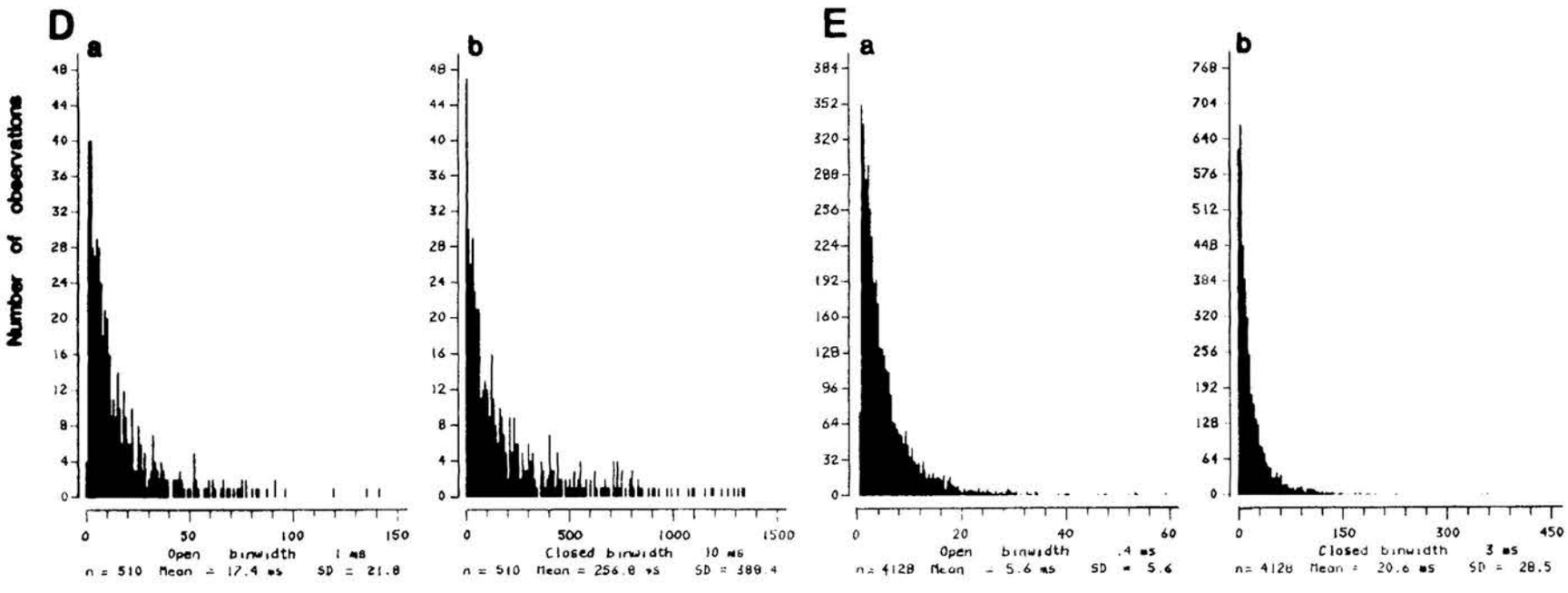
3.2 The effect of temperature on channel current activity in zona-free mouse eggs

Figure 3.1A is a pen trace showing channel current activity recorded at room temperature (22°C) in a zona-free mouse egg, bathed in Ca4K5. The patch pipette was

Figure 3.1

A and B are pen trace recordings of channel activity. A was recorded whilst the egg was bathed in Ca4K5 at 22°C i.e at room temperature. In B (the same egg as in A) the temperature of the bathing solution was 34-35°C at time zero. There was a decline in temperature of the bathing solution in B with time, since at time zero the heating coil was switched off. Asterisks in B refer to the "bursts" of inward currents. Ca and Cb are oscilloscope traces of outward currents recorded at 22°C and 34-35°C respectively. "S", "L", "S_1" and "L_1" refer to the amplitudes of channels observed (see text). Distributions of open and closed times at 22°C are shown in Da and Db respectively, and those at 34-35°C are shown in Ea and Eb respectively. The patch pipette was filled with Sr80K5. C, D and E were filtered at 600Hz (-3dB).


A
10pA
10 sec
B
Current (pA)
out
in
10
0
10
0
0.5
1.0
1.5
2.0
Time (mins)
C
a
b
L
S
C
L_1
S_1
4pA
40ms


D
a
b
Number of observations
Open binwidth 1 ms
n = 510 Mean = 17.4 ms SD = 21.8
Closed binwidth 10 ms
n = 510 Mean = 256.8 ms SD = 388.4
E
a
b
Open binwidth .4 ms
n = 4128 Mean = 5.6 ms SD = 5.6
Closed binwidth 3 ms
n = 4128 Mean = 20.6 ms SD = 28.5

filled with Sr80K5, and the transpatch potential was equal to the resting potential of the egg. Outward currents were observed (also shown as oscilloscope traces in Figure 3.1Ca), the open and close time distributions of which are shown in 3.1Da and 3.1Db respectively (percentage open time = 6%). In five experiments the bathing solution was warmed to a temperature of 34-35°C, and then the heating coil (which was immersed in the bathing solution) was switched off, indicated by time zero. Subsequently the temperature of the bathing solution declined. One such experiment is shown in Figure 3.1B (recorded in the same egg as in 3.1 A). The outward channel activity was greater (percentage open time = 21%), when the temperature of the bathing solution was 34-35°C. Outward channels recorded in the "warm" solution are also shown as oscilloscope traces in 3.1Cb, and their open and closed time distributions in 3.1Ea and 3.1Eb respectively. The mean open times at 22°C and at 34-35°C were 17.4ms and 5.6ms respectively. The mean closed times at 22°C and at 34-35°C were 256.8ms and 20.6ms respectively. There was approximately a tenfold increase in the number of openings in warm solution compared to those at room temperature.

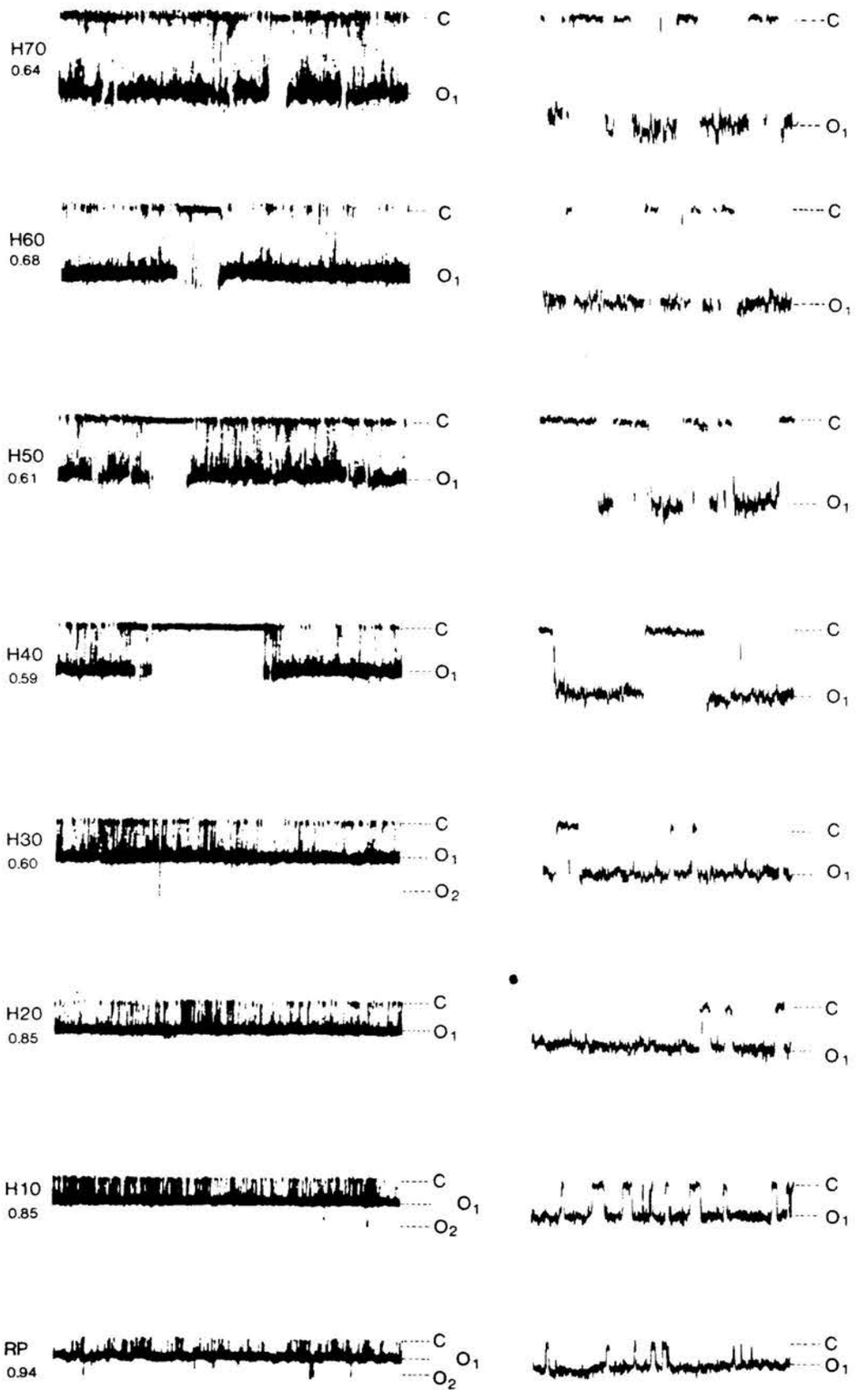
In warm solution two bursts of inward currents (denoted by downward deflections in 3.1B and marked by asterisks) were also observed. The activity in warm bathing solution declined with time. One possible cause of this may be the fall in temperature of the

bathing solution (because the heating coil had been switched off at time zero, at which time the recording was started). There appeared to be two amplitudes of outward currents at each temperature of bathing solution. At room temperature the amplitudes were 0.9 and 1.3pA (marked by "S" and "L" in 3.1Ca respectively) and in the warm solution they were 1.2 and 1.8pA (marked by "S_1" and "L_1" in 3.1 Cb respectively). These are the mean amplitudes of the two types of channels observed at each temperature. All amplitude measurements were made (from oscilloscope traces such as those in 3.1C) on channel openings of durations greater than 10msec. Therefore it is unlikely that these measurements were in error because of the limited frequency response of the recording apparatus. The range of channel amplitudes (for each group S,L,S_1 and L_1) was equal to the mean $\pm$ 0.2pA. It is not possible from the available results to conclude whether "S" is the same type of channel (i.e. same ion carrier at the two different temperatures) as either "S_1" or "L_1" and similarly whether "L" is the same type of channel as either "S_1" or "L_1". The only conclusion possible is that there are two amplitudes of outward channels at each temperature (and there is also an inward channel recorded when the egg is bathed in warm solution). Since the error in the measurement could be as large as $\pm$ 0.2pA, and the range was equal to the mean $\pm$ 0.2pA, the variance was not calculated.

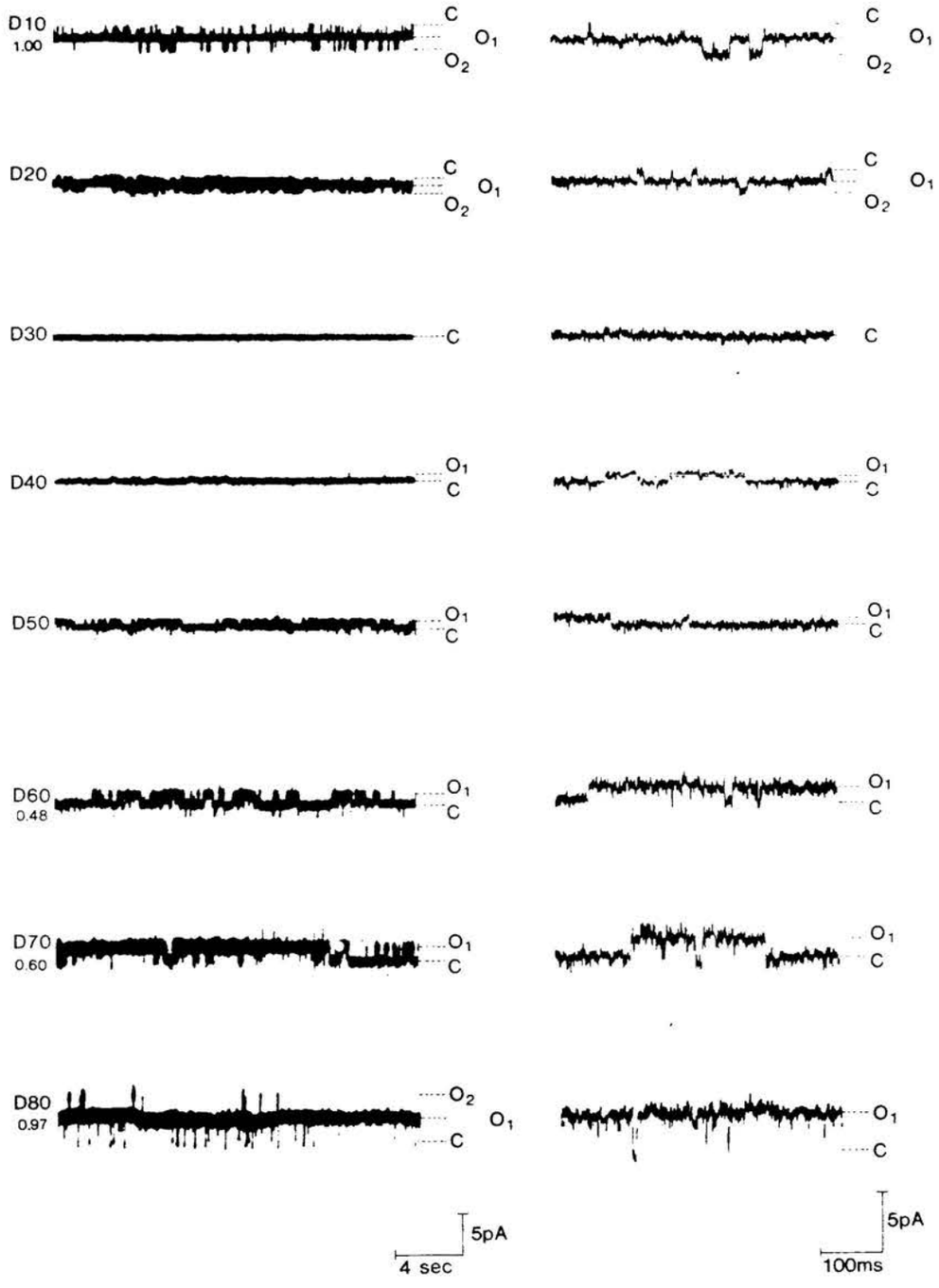
Figure 3.2

A and B are records of channel openings at different transpatch potentials (indicated to the left of each pen trace recording). At each transpatch potential (between H70 and D80) a pen trace record (left) and an oscilloscope trace (right) is illustrated. Numbers given below the transpatch potentials are the respective fraction open times. A value of 1.00 being equivalent to one channel being open all the time. For example, with a transpatch potential D10, the fraction open time has been denoted as 1.00 because, although one channel was not open all the time, occasionally two channels were open. In this case the time during which no channels were open was equivalent to the time during which two channels were open (hence fraction open time equals 1.00). All pen traces in A and B are at the same gain, and all the oscilloscope traces in A and B are at the same gain. All records in A and B were obtained in a cell attached patch on an egg bathed in Ca4K5 + La2. The pipette was filled with K155+EGTA. The oscilloscope traces were filtered at 350Hz (-3dB).

A


H70
0.64
H60
0.68
H50
0.61
H40
0.59
H30
0.60
H20
0.85
H10
0.85
RP
0.94
C
O1
O2

B


D10
1.00
D20
D30
D40
D50
D60
0.48
D70
0.60
D80
0.97
C
O1
O2
5pA
4 sec
5pA
100ms

Although in four other experiments, on four different eggs, an increase in channel activity was observed with an increase in temperature, such a preliminary analysis of channel amplitudes at each temperature was not possible.

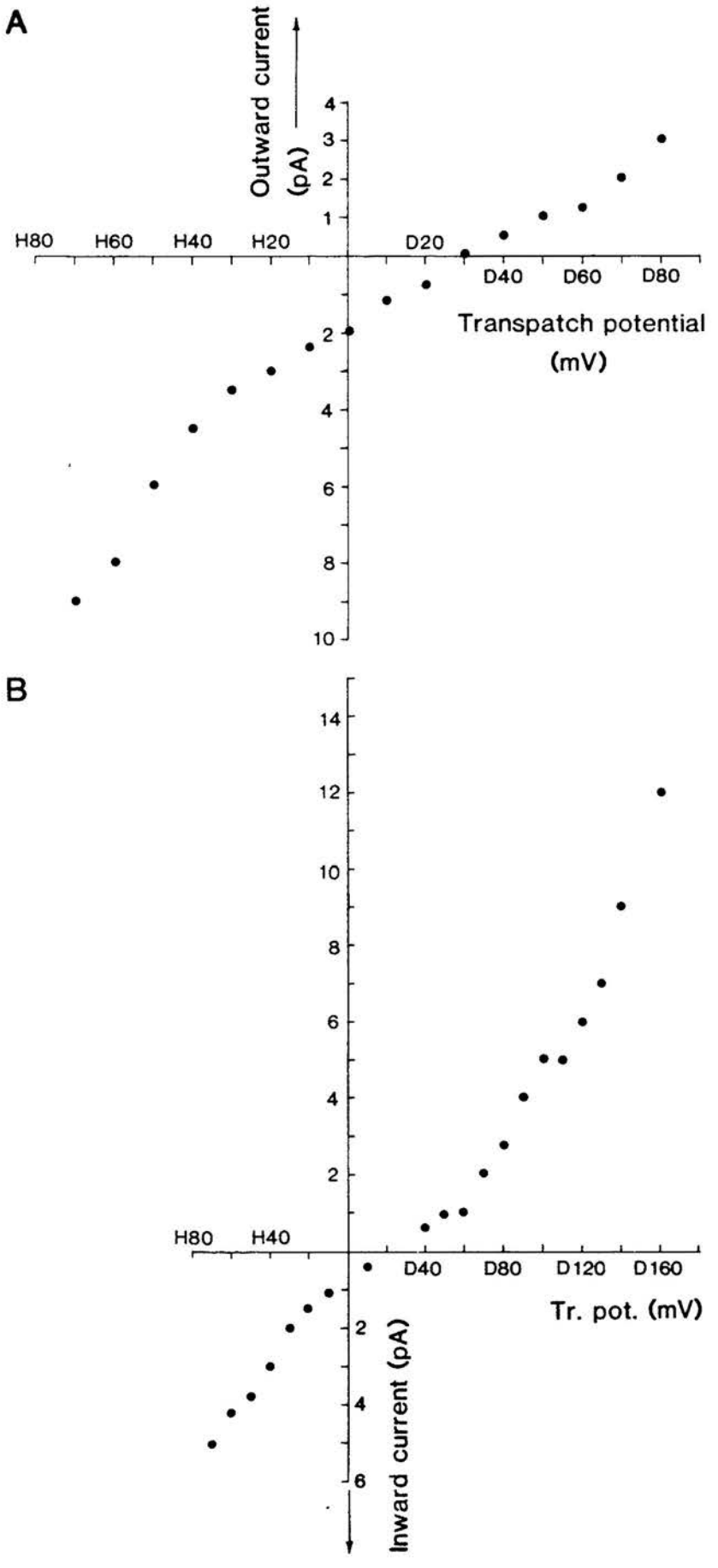
3.3 Potassium channels in zona-free mouse eggs

In six cell attached patches, on eggs bathed in normal solution containing 2mM lanthanum chloride and 4mM calcium chloride (Ca4K5+La2), channels which are believed to be potassium were observed. The patch pipette in these experiments was filled with K155+EGTA. These channels recorded at different pipette potentials are illustrated in Figure 3.2 (A and B - both groups of records obtained in the same cell attached patch). There appeared to be no subconductance state. The reversal potential of these channels was found to be D30 (i.e. transpatch potential is equivalent to the membrane potential depolarized by 30mV). For six such experiments the mean reversal potential was found to be D23±8mV (mean ± SD, n=6).

Since it is assumed that there is equal potassium on either side of the patch, then if these channels are potassium channels, they would be expected to reverse at a transpatch potential of 0mV. The latter is equivalent to D23±8mV and therefore the resting potential of these eggs can be estimated to be -23±8mV. This is assuming that at a zero pipette potential, the transpatch potential is equal to the resting potential

Figure 3.3

A. Current voltage relationship of the channels shown in Fig.3.2. The reversal potential is D30. B Current voltage relationship of channels recorded in another cell attached patch (egg bathed in Ca4K5 + La2, and the pipette filled with K155 + EGTA). In both A and B., the ordinate scale is the single channel current amplitude and the abscissa is the transpatch potential. The latter is referred to in terms of depolarization (D) or the hyperpolarization (H) of the resting potential produced by the imposed pipette potential.


A
Outward current
(pA)
4
3
2
1
H80
H60
H40
H20
D20
D40
D60
D80
Transpatch potential
(mV)
2
4
6
8
10
B
14
12
10
8
6
4
2
H80
H40
D40
D80
D120
D160
Tr. pot. (mV)
2
4
6
Inward current (pA)

of the egg.

Figure 3.3A is a plot of the current voltage relationship of the channels shown in Fig.3.2. It appeared to have two phases. The first phase between H70 and H30 was "steeper" (conductance given by the slope of the graph was 148pS) than that between H30 and D80. The conductance during this second phase was 59pS, i.e. this channel showed inward rectification, because it had a higher conductance for inward current (between H30 and H70) than for outward current (between H30 and D80). In similar experiments, even larger depolarizations of the transpatch potential, showed that these channels rectify also in the outward direction (as illustrated by Fig3.3B) also. Current voltage relationships for these channels, therefore appeared to show three phases. For example in Figure 3.3B, the first phase occurs from approximately H70 to H20 ("H" phase), the second phase between H20 and D50 ("P" phase) and the third phase is from D50 to D160 ("D" phase). The mean conductances of the H, P and D phases were 90±31pS (n=6), 41±10pS (n=6) and 121±38pS (n=5) respectively.

In four cell attached patches on eggs bathed in Ca10K5, the reversal potentials were D21, D24, D40 and H6 and the conductances were 152pS, 140pS, 84pS and 120pS respectively (the latter is discussed with reference to Figures 3.5 and 3.6). In all cases the pipettes were filled with K155 + EGTA. Using the same

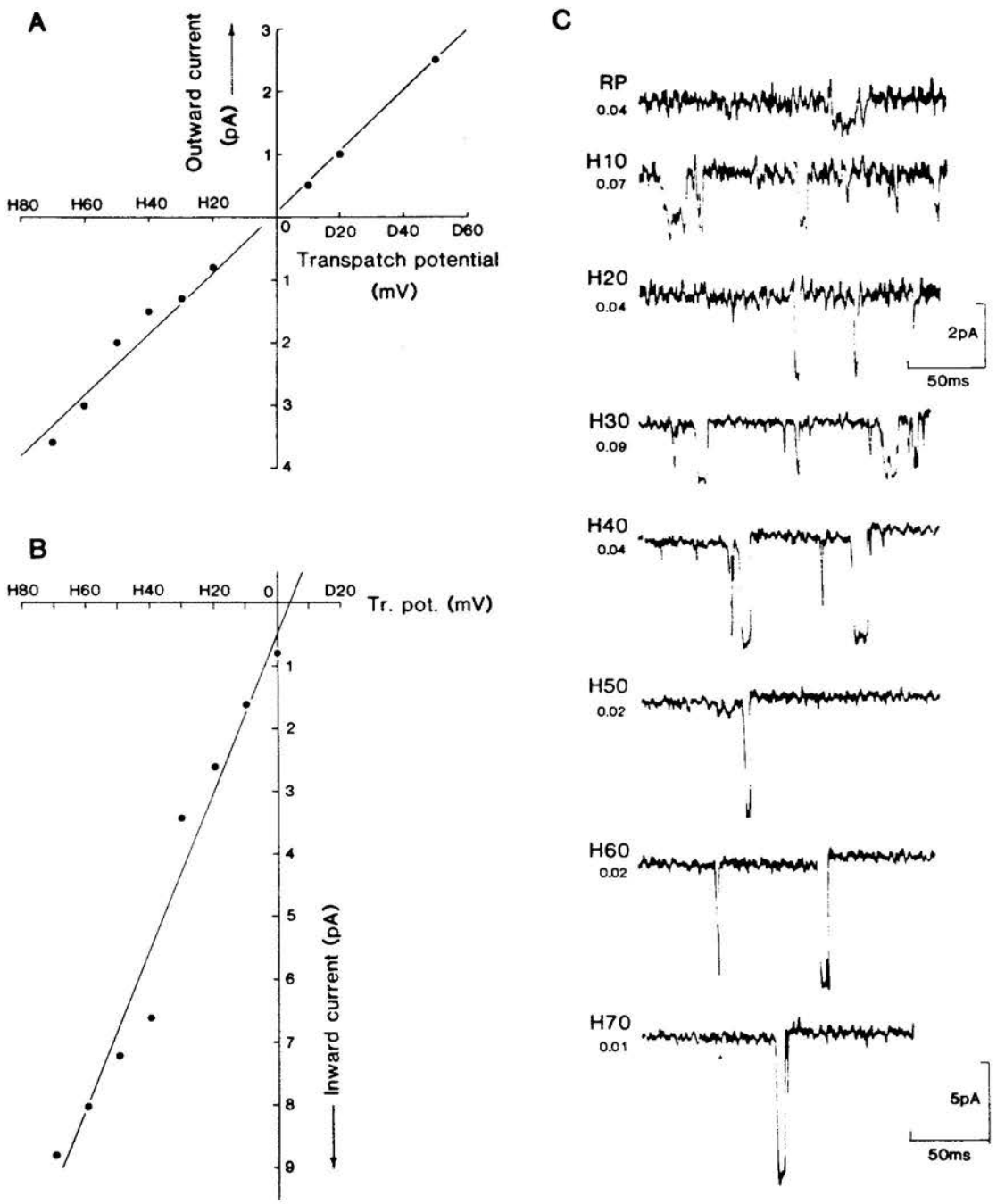
logic as above, the estimated resting potentials of these eggs are -21, -24, -40 and +6mV respectively.

There was no correlation between the transpatch potential and the percentage open time. For example, the channels illustrated in Figure 3.2 were open for between 59 and 94 per cent of the time, at transpatch potentials between H70 and RP. Whereas in other experiments, over the same range of transpatch potentials, the channels were open for between 0 and 11 per cent of the time. Similarly there was no correlation between depolarizing transpatch potentials (D10-D170) and the percentage open time. Although there were indications in three cell attached patches in three eggs, that an increasingly depolarized transpatch potential caused an increase in the percentage open time (e.g. in Fig.3.2 the fractional open times at D60, D70 and D80 were 0.48, 0.60 and 0.97). In another cell attached patch the fractional open time increased from 0.71 at D50 to 0.98 at D80. In two out of three cell attached patches these channels showed a significant correlation between the transpatch potential and the mean channel open time ($r=0.67$, $n=13$, $p<0.05$ in the range H70 - D80; and $r=0.80$, $n=10$, $p<0.01$ in the range H50-D100). These results suggest that the channel open times might be voltage sensitive.

The above results yield no conclusive information about what ion is passing through the channels. It cannot be sodium because no sodium was present in the

Figure 3.4

A and B. Current voltage relations for channels recorded in two different cell attached patches. On the ordinate is plotted the single channel current amplitude and on the abscissa the transpatch potential. The pipette and bath solutions for the results shown in A were K155 + EGTA (diluted to 25%) and Ca4K5 + La2 respectively. Similarly for those in B the pipette solution was K155 + EGTA modified to contain 78mM KCl and 78mM NaCl, and the bath solution was again Ca4K5 + La2. Oscilloscope traces of the results which are plotted in B are shown in C. To the left of each trace is written the respective transpatch potential and the fractional open time (value of 1.00 is indicative of a channel opening for 100% of the record). Note that the gain of the top three traces is greater than that of the remainder. All traces were filtered at 350Hz (-3dB).


A
Outward current
(pA)
3
2
1
H80
H60
H40
H20
0
D20
D40
D60
Transpatch potential
(mV)
1
2
3
4
B
H80
H60
H40
H20
0
D20
Tr. pot. (mV)
1
2
3
4
5
6
7
8
9
Inward current (pA)
C
RP
0.04
H10
0.07
H20
0.04
2pA
50ms
H30
0.09
H40
0.04
H50
0.02
H60
0.02
H70
0.01
5pA
50ms

pipette filling solution (the solution was buffered with potassium hydroxide, whereas normal solution during this study was buffered with sodium hydroxide). It is unlikely to be calcium because the pipette contained only 10^{-9}M calcium (buffered with EGTA). The two possible remaining candidates were investigated, i.e. potassium and chloride ions.

In one group of experiments (7 cell attached patches on seven eggs) the possibility that the channel currents were due to movement of anions was ruled out. In these experiments the pipette filling solution (K155+EGTA) was diluted to either 80%, or 50% or 25% with glass distilled water. The bathing solution in all these experiments was Ca4K5 + La2. This was diluted to 80% in experiments in which the pipette filling solution had been diluted to 50% or 25%.

A current voltage relationship of the channels observed in a cell attached patch (pipette filled with 25% K155+EGTA) is shown in Fig.3.4A. In this relationship there were no distinct phases, indeed the single channel current amplitude increased in a linear manner with increasingly depolarized transpatch potential (correlation coefficient = 0.99, n=9). The conductance was 49pS and the reversal potential obtained by extrapolation from the graph in Fig.3.4A was about H1. In another similar experiment the reversal potential was H5 and the conductance was calculated to be 40pS (current voltage relationship

also linear, between transpatch potentials H50 and D50).

From these two results, it is evident that the reversal potential for these channels was more negative when the pipette solution was diluted (in the undiluted solution the reversal potential was D23±8mV - see above). Such a shift would only be expected for a cationic channel (dilution of the pipette solution would have been expected to produce a positive shift in the reversal potential had it been an anionic channel).

The concentration of potassium and chloride in K155+EGTA was 157mM and 159mM respectively whereas in pipette solutions diluted to 25% it was 39mM and 40mM respectively.

The assumption is made in these experiments that the resting potential of zona-free mouse eggs is unaffected by altering the composition of the pipette filling solution. It is possible that the resting potential of the eggs was affected by bathing the eggs in a diluted solution (80% in the above two experiments). It would be interesting to "patch" a given egg repeatedly with different pipettes containing solutions diluted to different extents. This was not done because it was felt that the eggs deteriorated whilst in the recording chamber (i.e. their membrane potential was not constant - see Chapter 4).

In an experiment in which the pipette solution was diluted to 50% the reversal potential was D9 (more hyperpolarized than that for the undiluted solution)

and the conductance was 85pS (correlation coefficient = 0.99, n=9).

Results obtained in pipette solutions diluted to 80% were similar to those in the undiluted solutions. In such experiments the reversal potentials were D30, D32, D35 and D36mV and the H, P and D phases were again observed. The conductance of the H phases in three of the patches were 125, 64 and 80pS and those of the P phases were 27, 22 and 26pS. A value for the conductance of D phase was only obtained for one patch, i.e. 74pS.

The above experiments indicated that the channels are cation selective, and due to the composition of the pipette solution it seemed very likely that the channels were potassium channels. In the experiments now described, the potassium concentration in the pipette solution was lowered by substituting sodium chloride for potassium chloride on an equimolar basis (hence the chloride concentration was maintained constant). The composition of the pipette filling solution was the same as K155 + EGTA, except that the concentration KCl was 78mM, as was that of NaCl. The results of one such experiment (3 such experiments were done in total) are illustrated in Fig.3.4B. The bathing solution was Ca4K5+La2. The current voltage relationship (3.4B) was found to be linear (correlation coefficient = 0.98, n=8), and the conductance was 125pS. The reversal potential, obtained by

extrapolation of the line of best fit in Fig.3.4B was D4. The scatter of the points on the graph, indicates that there may be an error of about ±5mV, in this measurement of the reversal potential. Even assuming the extreme possibility, that the reversal potential is underestimated by 5mV, the value obtained by correction (i.e. D9) is still more negative than that obtained when the pipettes were filled with K155+EGTA. Since the potassium in the pipette filling solution has been halved (from 155mM to 78mM), the reversal potential for potassium channels is expected to be -18mV (i.e. 60 log 0.5). This is still assuming that the cytosolic potassium concentration is 155mM (the temperature of the bathing solution is 34°C). Figure 3.4B shows that the channels reverse at D4, which must be equivalent to a transpatch potential of -18mV. Hence assuming that at zero pipette potentials the patch pipette does not alter the resting potential of the egg, the latter must be equal to -18-[+4] = -22mV. Oscilloscope traces of the channel records are shown at the 8 transpatch potentials in Fig.3.4C.

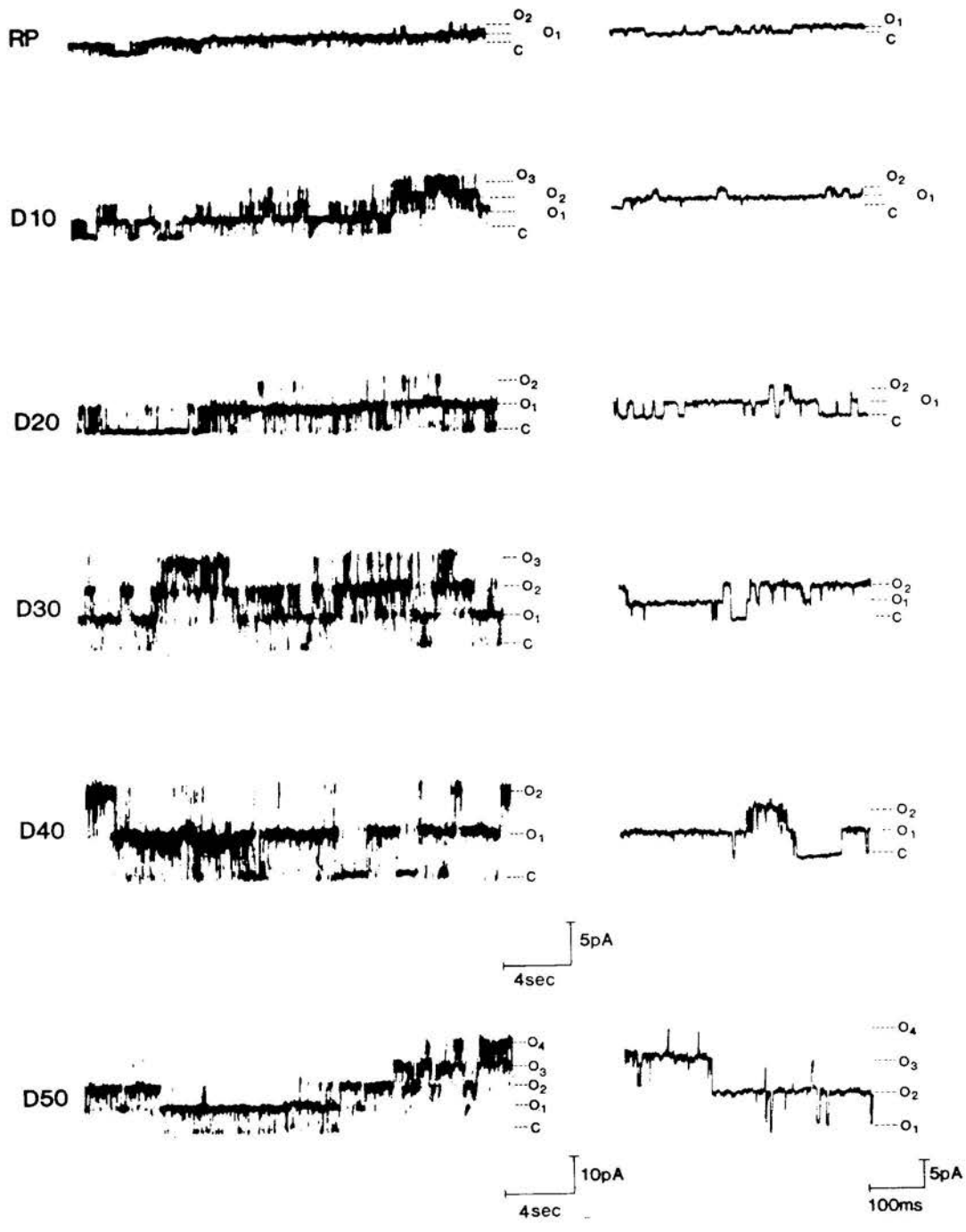
In another similar experiment the reversal potential was D5 (making the same assumptions as above the resting potential of this egg must be -23mV) and the channel conductance equal to 157pS. Hence in both these experiments the reversal potential was more hyperpolarized than in experiments in which the pipette was filled with K155+EGTA, indicating a cationic channel (in this case potassium).

For the results shown in Fig.3.4B, there was no correlation between the transpatch potential and the mean open time ($r=0.09$, $n=8$; range equals 1.7-3.9ms). But the mean closed time increased as the transpatch potential was hyperpolarized ($r=0.77$, $n=8$, $p<0.05$; range equals 41.0-458.7ms). Also the percentage open time decreased from 9 per cent (at H30) to 1 per cent (at H70).

To summarize, when the pipette was filled with a solution containing a potassium concentration resembling that of the egg's interior, then during small depolarizations or hyperpolarizations (about H30 to D50) the potassium channel had a conductance of about 40pS. For larger depolarizations and hyperpolarizations the channel apparently rectified (outward and inward respectively), having at such potentials conductances as high as 160pS approximately. The possibility of the channel being a chloride channel has been ruled out. The channels had a reversal potential which was about 20mV more positive than the resting potential of the egg, when the patch pipette contained 155mM potassium. At such potassium concentrations in the pipette the reversal potential for potassium channels is expected to be 0mV (since in this situation we have an assumed that there is equal potassium concentration on either side of the patch membrane). Hence the depolarization of the transpatch required to get reversal of the channels should be

Figure 3.5

Pen traces (left) and oscilloscope traces (right) of multiple potassium channel openings recorded at different transpatch potentials. All the oscilloscope traces (filtered at 350Hz, -3dB)are at the same gain, but the pen trace recorded at a transpatch potential of D50 is at a different gain to the remainder. Up to five channels were detected in this patch, but a maximum of only four openings is shown for the record at D50.


RP
D10
D20
D30
D40
D50
O2
O1
C
O3
O4
5pA
4sec
10pA
4sec
5pA
100ms

equal to the actual membrane potential across the patch without depolarization.

3.4 Do the potassium channels open and shut independently of one another?

If the "n" channels in a membrane patch have similar properties and act independently of one another, then the percentage of time P(r), that 0,1,2,_ _ _ channels are open should be described by a binomial distribution such that

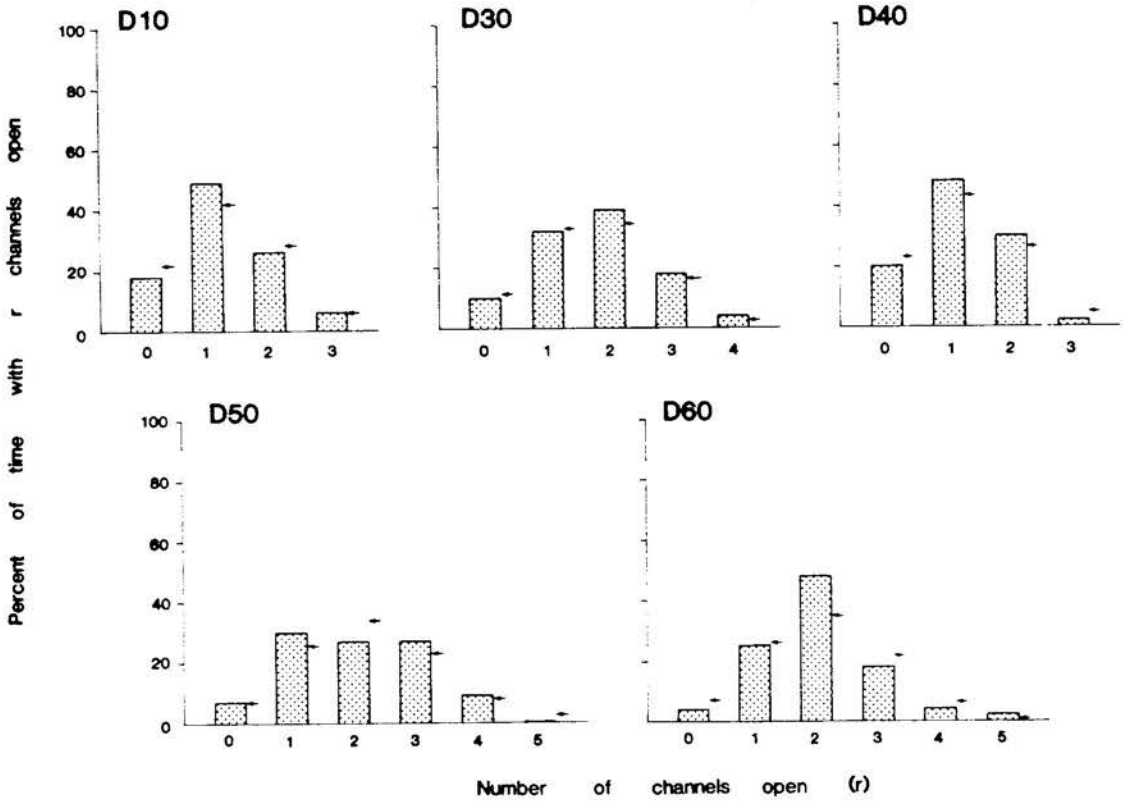
$$P(r) = 100 \frac{n!}{r!(n-r)!} p^r (1-p)^{n-r} \qquad (1)$$

where n is the number of channels in the membrane patch, r is the number of channels open, and p is the probability that any given channel is open (Colquhoun, 1971).

The previous section described results from cell attached patches in which there was predominantly one channel opening. In Fig.3.5 are illustrated multiple channel openings in a cell attached patch on an egg bathed in Ca10K5. The pipette was filled with K155 + EGTA. For each record shown in Fig.3.5, the percentage of total recording time that only 0,1,2 _ _ _ n channels were open was calculated. These are the observed values and are plotted as bars in Fig.3.6. The probability (p) of any channel opening was then calculated as follows. Let the percentage of total

Figure 3.6

Plots of percentage time during which 0,1,2,3,4 or 5 channels were open for a cell attached patch with five channels. Each plot is at a different transpatch potential. The observed values (bars) and the predicted values (arrows) calculated from the binomial distribution are in close agreement, suggesting that the channels opened and closed independently of one another. The plots are of the channels which were displayed in Fig.3.5. The conductance of these channels was 120pS (r=0.97, n=8) and the reversal potential was H6mV. This value of the reversal potential is in disagreement with values obtained from other similar experiments (3.3).


D10
D30
D40
D50
D60
100
80
60
40
20
0
0 1 2 3
0 1 2 3 4
0 1 2 3
0 1 2 3 4 5
0 1 2 3 4 5
Percent of time with r channels open
Number of channels open (r)

recording time spent with 0,1,2 _ _ _ n channels open be $T_o, T_1, T_2 - - - T_n$. Then

$$p = \frac{(T_o x0)+(T_1 x1)+(T_2 x2) \_ \_ \_ +(T_n xn)}{nx100} \qquad (2)$$

From the value of p so obtained (equation 2), the predicted percentage open time in each open state was calculated, from the equation for a binomial distribution given at the beginning of this section (equation 1). The value of p used for the calculation of the predicted values at each transpatch potential were 0.401 for D10, 0.413 for D30, 0.378 for D40, 0.402 for D50 and 0.393 for D60. These predicted values are indicated by arrows in Fig.3.6. The distribution of time spent with 0,1,2 _ _ n channels open, was close to that expected if the five (maximum number of channel openings in this patch) channels in the patch had similar properties and opened and closed independently of one another. Results consistent with independent channel activity were also observed in two additional experiments in which the number of channels in the patch was two and three.

Similar predicted values were obtained if p was calculated using equation (3). This was obtained by substituting r=0 in equation 1 (i.e. percentage of recording time during which no channels were open in the patch).

$$P(0) = 100 \frac{n!}{0!n!} p^{o}(1-p)^{n}$$

$$= 100\ (1-p)^n \qquad (3)$$

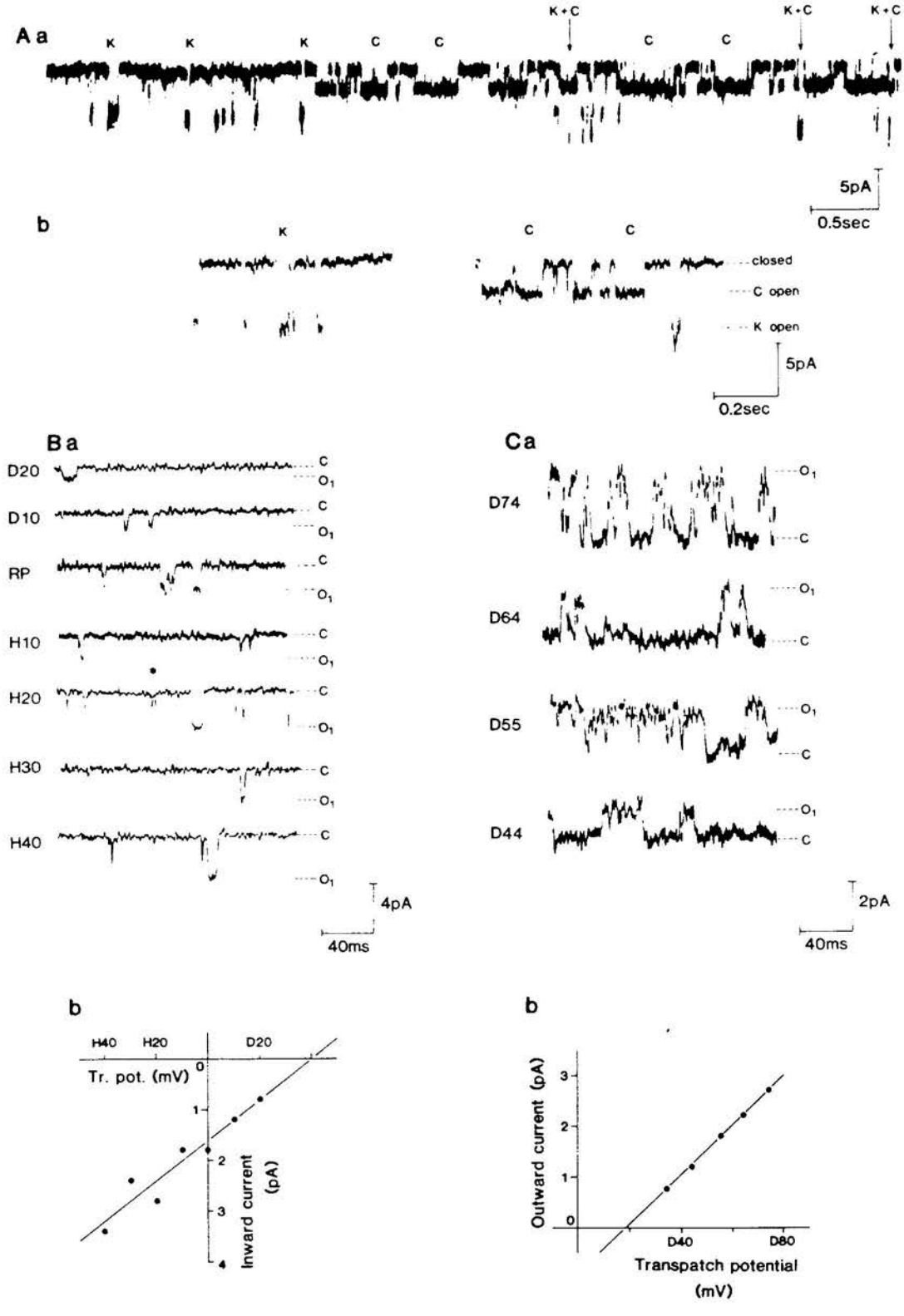
where n = number of channels in the patch.

3.5 Possible chloride channels in zona-free mouse eggs

During cell attached patch recording in eggs bathed in Ca4K5+La2 (pipette filled with K155+EGTA), with hyperpolarized transpatch potentials, occasionally two distinct sizes of inward channels were observed. One such example is shown in Fig.3.7A. In this experiment the transpatch potential was H70 and the amplitudes of the two channels were 6pA and 2.5pA. The former is a potassium channel (like the ones discussed in the previous two sections) and the latter is believed to be either a chloride or a second type of potassium channel. Examination of oscilloscope traces revealed that the amplitude of the large channel was not equal to twice that of the small channel. Hence a large channel opening cannot be interpreted as being two small channels opening simultaneously. The smaller amplitude channel is unlikely to be a subconductance state of the larger channel, because during the whole record there was no evidence of two large channels opening simultaneously, even though amplitudes equal to the sum of small and large channels were recorded. In Fig.3.7A (a is a pen trace and b are two oscilloscope traces) the potassium channel is denoted by "K" and the possible chloride channel opening by "C", and a simultaneous opening of the two as "K+C". The small

Figure 3.7

Aa Pen trace record of inward potassium (K) and possible chloride (C) channels. Oscilloscope pictures of these channels are shown in Ab(filtered at 350Hz, -3dB). In this experiment the egg was bathed in Ca4K5 + La2 and the pipette was filled with K155 + EGTA. Ba and Ca are oscilloscope traces (all filtered at 350Hz, -3dB) of channels recorded at different transpatch potentials in two cell attached patches on different eggs. In both cases the eggs were bathed in Ca4K5 and the pipettes were filled with Sr80K5. Bb is a current voltage relation of the inward channels shown in Ba, and Cb is a current voltage relation of the outward channels illustrated in Ca. Ordinate: single channel current amplitude; abscissa: transpatch potential (mV). The asterisk above the trace recorded at H20 denotes a probable calcium channel opening (discussed in 3.6), at that transpatch potential.


A a
K
K
K
C
C
K+C
C
C
K+C
K+C
5pA
0.5sec
b
K
C
C
closed
C open
K open
5pA
0.2sec
B a
D20
D10
RP
H10
H20
H30
H40
C
O1
4pA
40ms
C a
D74
D64
D55
D44
O1
C
2pA
40ms
b
H40
H20
D20
Tr. pot. (mV)
0
1
2
3
4
Inward current (pA)
b
Outward current (pA)
3
2
1
0
D40
D80
Transpatch potential (mV)

inward currents are not due to sodium or calcium influxes, because sodium was not present in the pipette filling solution, and calcium was present at 10^{-9}M (buffered with EGTA; since the pipette was filled with K155+EGTA).

Figures 3.7Ba and 3.7Ca are oscilloscope traces of channel records at different transpatch potentials (in two eggs bathed in Ca4K5). The pipette filling solution in these experiments was Sr80K5. For the channels illustrated in Fig.3.7.Ba the reversal potential by extrapolation was D41mV (current voltage relationship shown in 3.7Bb), and for those shown in 3.7Ca (recorded in a different cell to those in 3.7Ba) the reversal potential by extrapolation was D19mV (current voltage relationship shown in 3.7Cb). As found by previous workers (Igusa & Miyazaki, 1983) and further supported by experiments later described in this study (Chapters 5 and 6), the reversal potential for a type of response due to a calcium activated potassium conductance was calculated to be about -80mV. If therefore these were potassium channels, then the potential difference across the patch prior to depolarization (i.e. pipette potential = 0mV) would be -121mV (i.e. -80-[+41]) in 3.7B and -99mV in 3.7C (i.e. -80-[+19]). In other words the resting potential of the egg in B was -121mV and that in C was -99mV (assuming that "patching" the egg does not affect the resting potential of the egg). Based on the assumption that cytosolic potassium is 155mM then the

estimations of the resting potential would be more negative by 9mV (since then the potassium equilibrium potential =-89mV). If these are indeed chloride channels, then assuming a chloride reversal potential of -60mV (concentration of chloride in the pipette was 167mM which is assumed to be ten times that in the cytosol) then the resting potential of the egg in B was -101mV and that in C was -79mV.

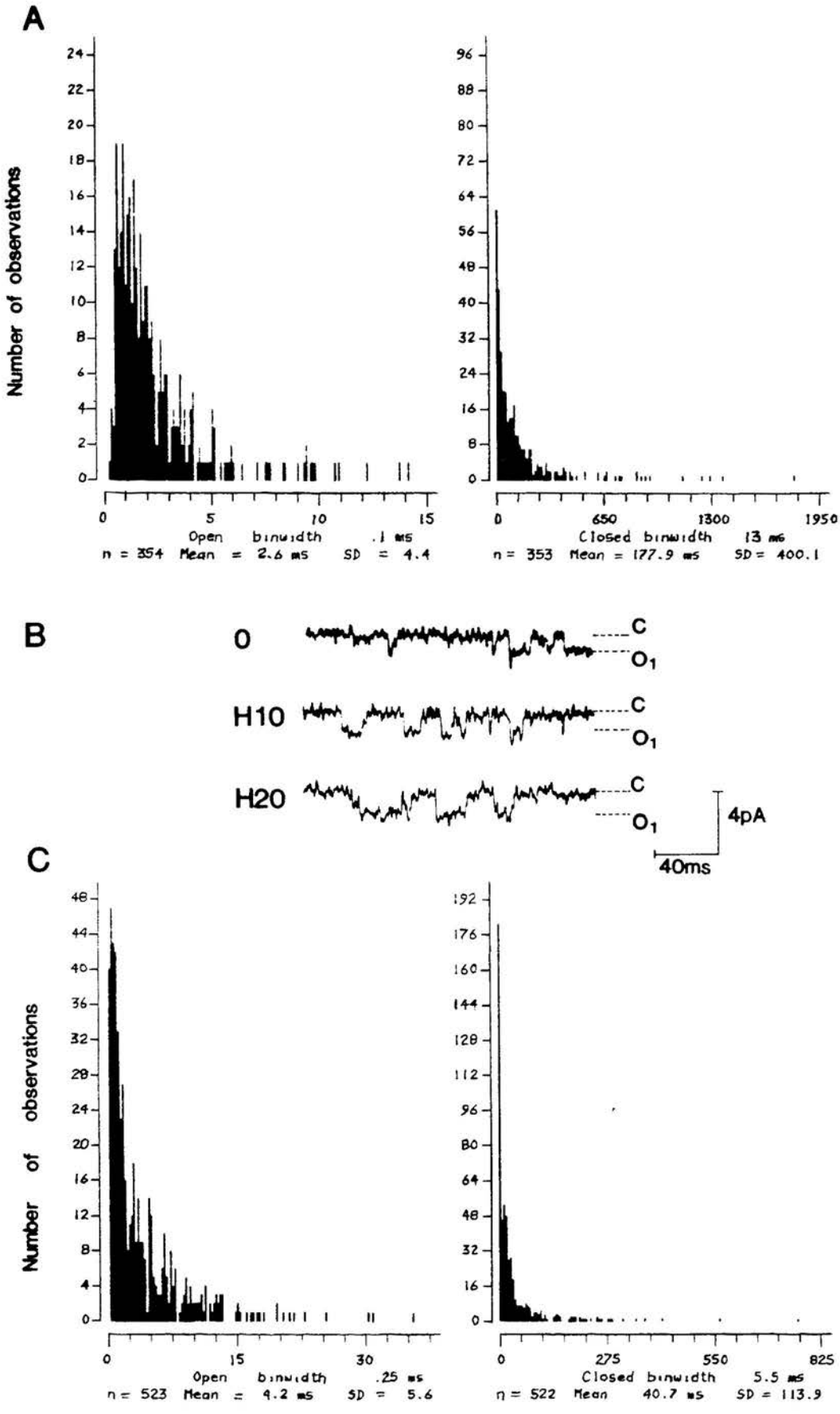
It is possible that the channels in 3.7B are calcium channels (since the patch pipette was filled with Sr80K5) which either reverse at about D41mV or are inactivated at transpatch potentials more positive than D20. It is unlikely that those in Fig. 3.7A, are calcium channels since in the latter the pipette was filled with K155+EGTA. If the channels illustrated in Fig. 3.7Ca are interpreted as being calcium channels (patch pipette filled with Sr80K5, hence the ion carrier through these channels would be strontium), then one would have to accept the fact that a depolarization of the transpatch potential by 19mV (see reversal potential in Fig. 3.7Cb), was sufficient to reverse these channels. From the work on calcium channels in other preparations, this seems unlikely.

The conductance of the channels shown in B was 40pS (r=0.95, n=7) and that in C was 50pS (r=1.00, n=5). There were no indications of rectification in these channels.

For the results illustrated in 3.7B, the

Figure 3.8

A and C. Open and closed time distributions for channels recorded in cell attached patches on eggs bathed in Ca4K5. In A the pipette was filled with Sr80K5 and the transpatch potential was H20, and in C the pipette was filled with Sr60/TEA/CsCl and the transpatch potential was D20. Examples of channels recorded in the same patch as those analysed and plotted in C, are given as oscilloscope traces in B at three different transpatch potentials.


A
Number of observations
Open binwidth .1 ms
n = 354 Mean = 2.6 ms SD = 4.4
Closed binwidth 13 ms
n = 353 Mean = 177.9 ms SD = 400.1
B
0
H10
H20
C
O1
4pA
40ms
C
Number of observations
Open binwidth .25 ms
n = 523 Mean = 9.2 ms SD = 5.6
Closed binwidth 5.5 ms
n = 522 Mean 40.7 ms SD = 113.9

percentage open time ranged from 0.23% to 1.44% (range of transpatch potentials from D20-H40). The range of mean channel open times was from 1.9 - 5.2ms. A plot of the open and closed time distributions for these channels observed at a transpatch potential of H20 is shown in Fig. 3.8A.

Such channels were also recorded in four experiments in which the pipettes were filled with Sr60/TEA/CsCl (Fig. 3.8B). The range of percentage open times and mean open times (Fig. 3.8C) was 1.59% to 9.77% and 2.4ms - 4.8ms over the range of transpatch potentials H30-D40. No correlation was observed between either the mean open time or percentage open time and the transpatch potential. In three out of these four experiments, the conductances of these channels were 20, 27 and 36pS, and their reversal potentials (by extrapolation of the I-V relations) were D29, D30 and D30mV. It is unlikely that these are potassium channels because of the caesium chloride and the TEA present in the pipette filling solution. But it is unfortunate that during these experiments the transpatch potential was not depolarized to values greater than D40, and hence no outward currents were recorded. It could therefore be argued that these channels are in fact calcium channels (the ion carrier being predominantly strontium, since the pipette filling solution contained 60mM$SrCl_2$ and 1mM calcium chloride) and that they became inactivated at potentials around D30.

3.6 Calcium channels in zona-free mouse eggs (Table 3.1)

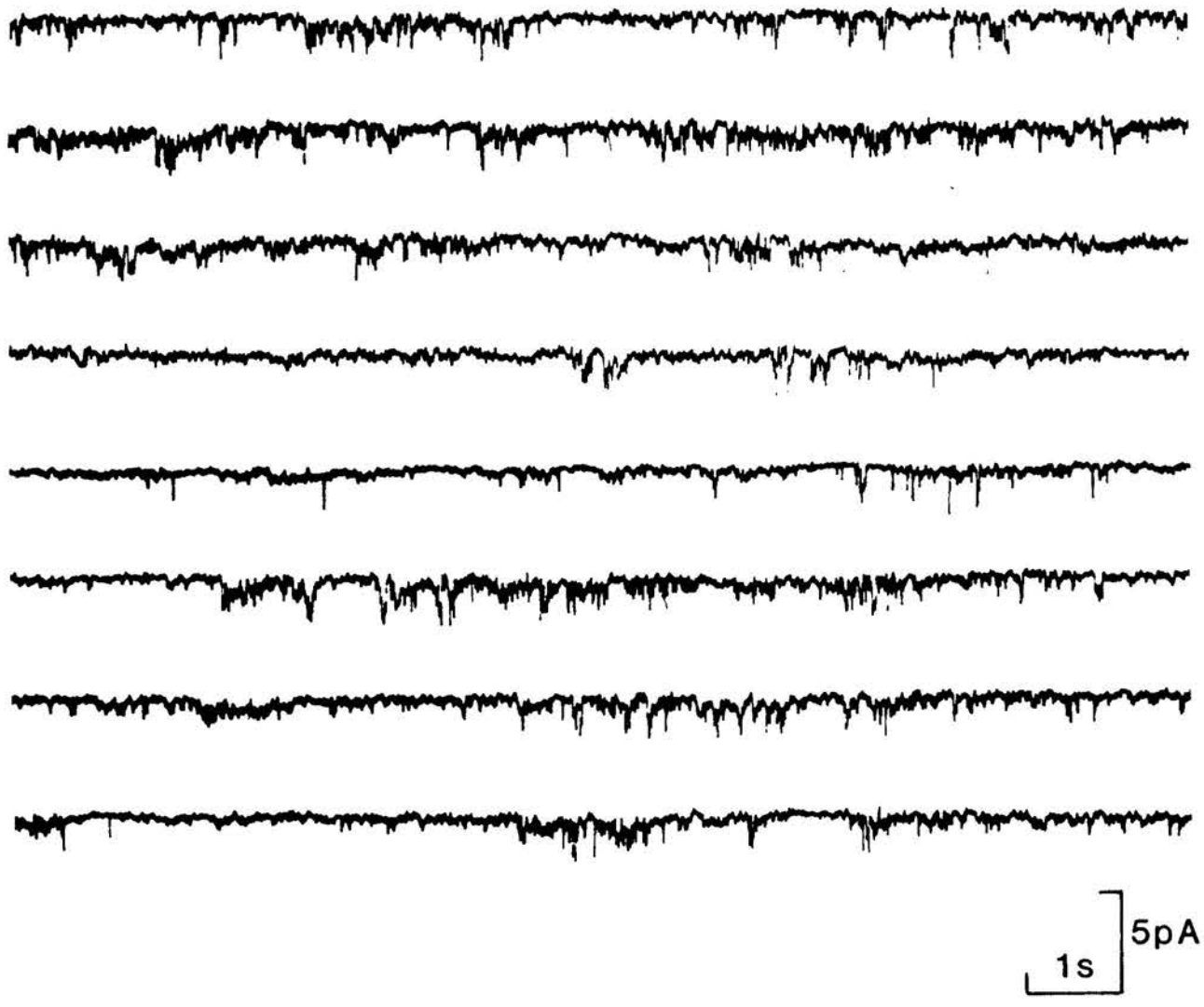
During this study no amplitude distribution histograms were plotted, but it was quite clear whilst analysing records such as those shown in Fig. 3.7Ba that such distributions would not have a sharp defined peak. One explanation offered for this is that more than one kind of channel was present. For example the channel opening marked by an asterisk (on trace recorded at a transpatch potential of H20) in Fig. 3.7Ba is probably a different type of channel to the possible chloride channels discussed in the previous section. It is believed that such small amplitude inward channels (about 1pA) with small open times (less than about 2ms) are calcium channels. Unfortunately their analysis has proved to be difficult. This is illustrated by the consecutive pen trace record shown in Fig. 3.9A. These records show a lot of inward channel activity, which occurs in bursts, with varying amplitudes of single channel currents. Some of these are likely to be the "possible chloride" channels discussed in the previous section and the remainder are probably calcium channels.

Possible calcium channels are marked by asterisks above the oscilloscope traces shown in Fig. 3.9Bb (in Fig. 3.9Ba are oscilloscope traces showing no channel openings, recorded in the same cell attached patch.)

Figure 3.9

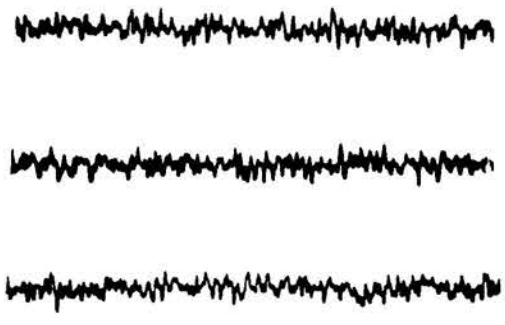
A Pen trace record of consecutive traces of inward channels recorded from a cell attached patch on an egg bathed in Sr80K5 at 24°C. The pipette filling solution was also Sr80K5 diluted to 90%. B Oscilloscope traces showing channel openings, marked by asterisks in b and traces showing no channel openings, a, recorded from a cell attached patch on an egg bathed in Ca4K5. The pipette filling solution was Sr60/TEA/CsCl. C. Consecutive pen trace record of inward channels (recorded under the same conditions as in A) believed to be predominantly chloride, but a calcium channel opening (duration approximately 22ms) is indicated by the arrow below the pen trace. The pipette potential was 0mV. D. Oscilloscope traces of two examples of outward channels immediately followed by inward currents (marked "O" and "I" respectively). The conditions of this experiment were also the same as those in A, except that the temperature of the bathing solution was 34°C.

A

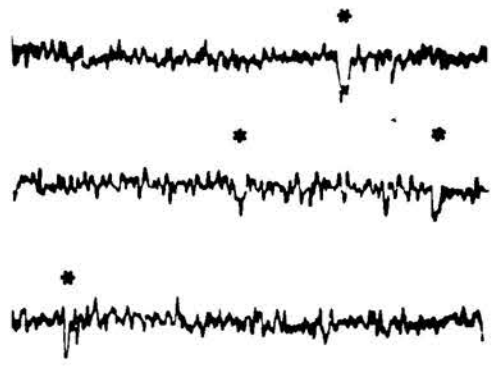


B

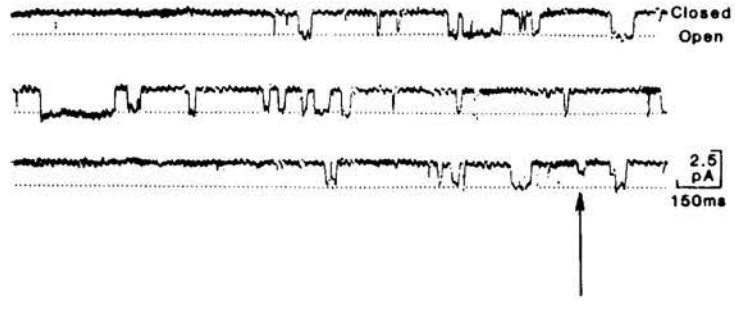
a



b



C



D

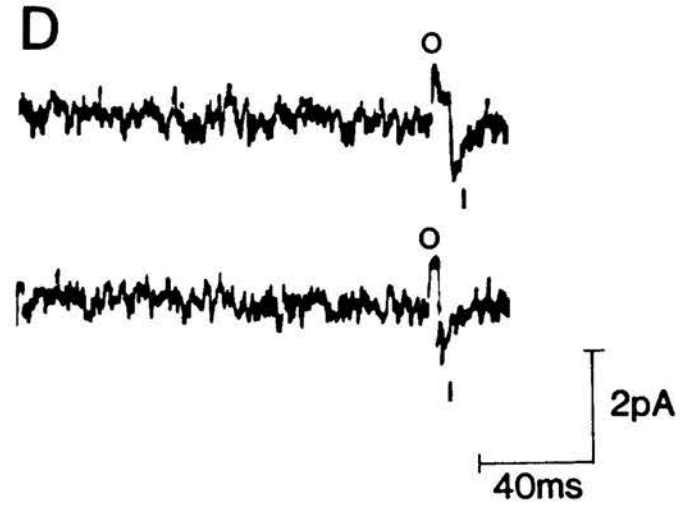


Table 3.1

A list of the measured calcium channel amplitudes, at different transpatch potentials. Four different pipette filling solutions were used.

Pipette filling solution	Transpatch potential	Calcium Channel Amplitude (pA)
Cal0K5 diluted to 90%	D25 H20 H60	1.1 0.8 0.9
Sr60/TEA/CsCl	D10 0(RP) H10 H20 H30	1.0 0.8,1.0,1.0,1.2,1.2,1.6 1.1,1.3,1.4 1.0,1.7 1.0,1.1,1.4
Sr80K5	D20 D10 0(RP) H10 H20 H30 H40 H70	1.2 1.1 0.6,1.0,1.0,1.0,1.1,1.3,1.7,1.8 1.0 1.0 0.3,1.6 1.2 0.6
90mM$SrCl_2$	0(RP)	0.7

TABLE 3.1

An example of a long duration calcium channel opening (about 22ms) is shown in 3.9C (marked by the arrow; amplitude = 0.6pA). Due to their small amplitudes and to the small number of occasions on which they could be measured accurately, a detailed analysis of calcium channels was not possible (i.e. current voltage relations, open and closed time analyses). Therefore actual values of single channel currents measured at different transpatch potentials have been tabulated (Table 3.1). The mean amplitude of these calcium channels was 1.10±0.33pA (mean ± SD, n=35).

In three cell attached patches it was possible to get an indication of the conductance of these channels, i.e. 7.5pS, 13pS and 20pS. In all three the pipette filling solution and the bathing solution was Sr80K5.

During two cell attached patch recordings, "coupled openings" (Fig. 3.9D) were observed. In both these cases the eggs were bathed in Sr80K5 and the pipette filling solution was the same only diluted to 90%. These openings, at zero transpatch potentials were outward currents immediately followed by inward currents, never vice versa. These could be either potassium or chloride outward currents followed by an inward calcium current.

3.7 Calcium, chloride and potassium channels in zona-free hamster eggs

All the experiments described so far in this chapter were performed on zona-free mouse eggs, the

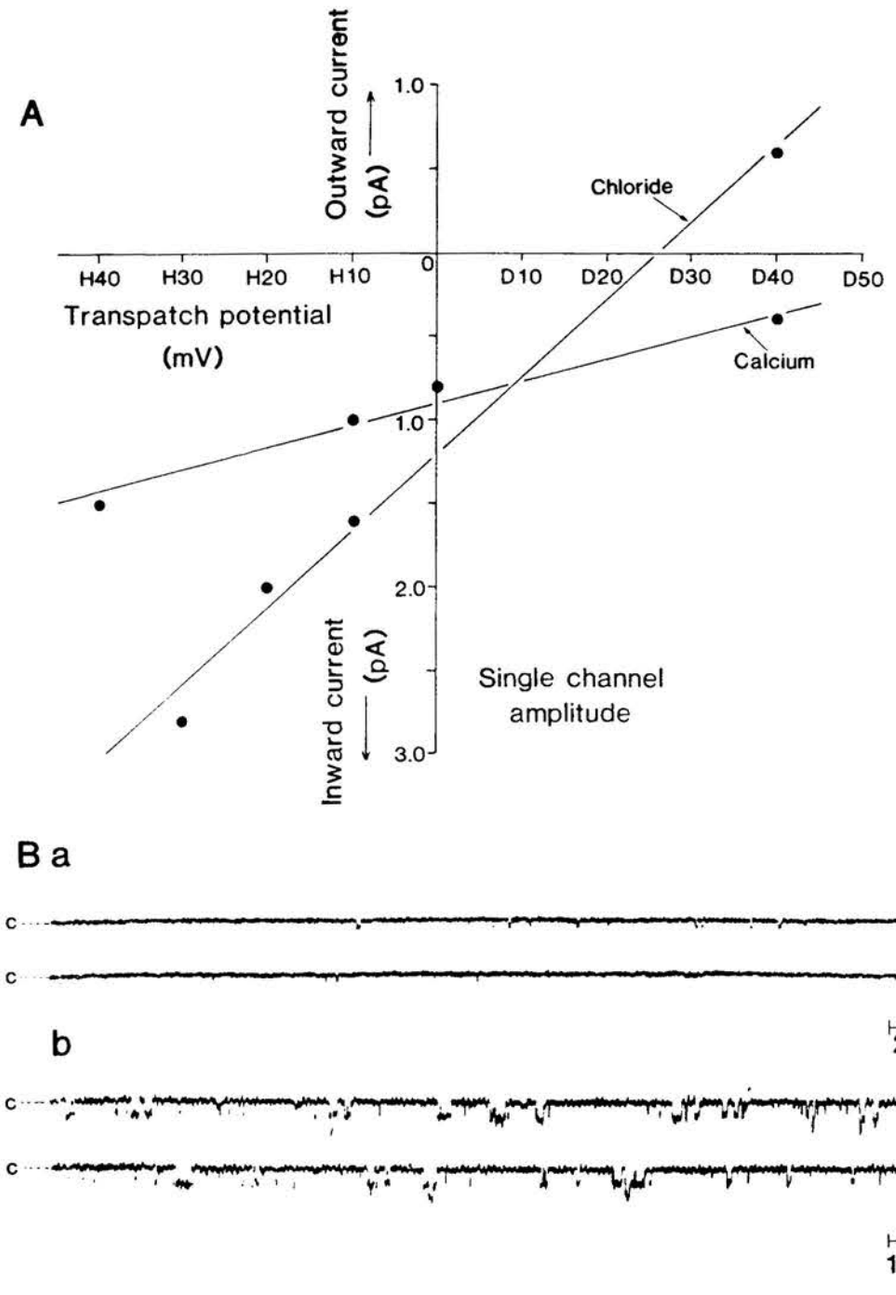
ones below are on zona-free hamster eggs.

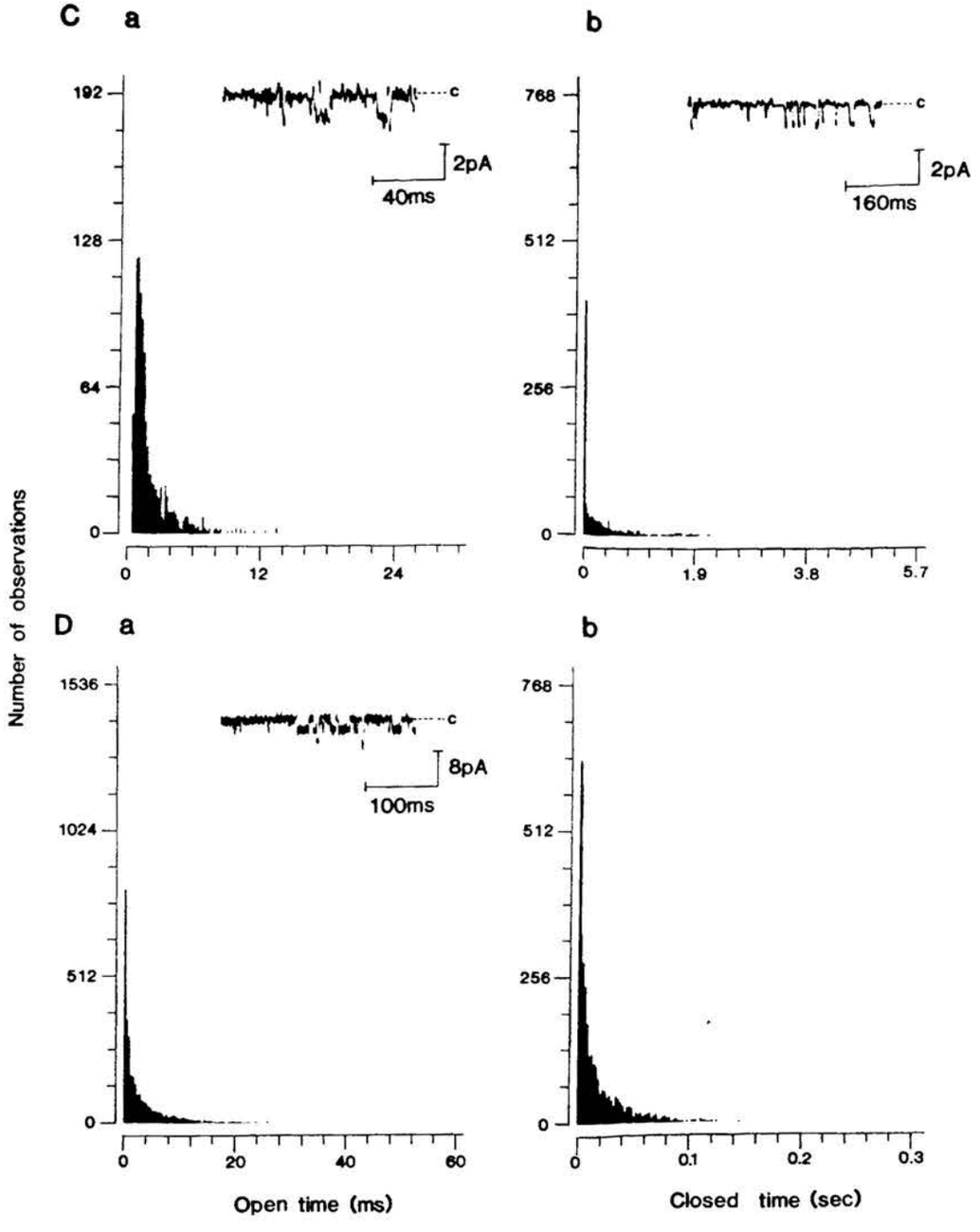
During a cell attached patch recording from an egg bathed in Ca10K5 (pipette filled with Sr60/TEA/CsCl) inward channels were observed at hyperpolarized transpatch potentials, which appear to fall into two categories on the basis of their amplitudes. When the measured amplitudes were plotted (Fig 3.10A), it was found that they could be "joined" by two lines, i.e. two groups of channels with differing conductances. The conductance of one group was 13pS (r=0.99, n=4; marked "calcium") and that of the other was 46pS (r=0.99, n=4; marked "chloride"). The former is believed to be a calcium channel and the latter a chloride channel (both of which have been discussed with reference to zona-free mouse eggs in 3.6 and 3.5 respectively). The reversal potential of the chloride channels was D26mV. An open and closed time distribution was not plotted since the total number of events was small. Due to the composition of the pipette filling solution, neither of these channels is likely to be a potassium channel.

During three of the experiments described in Chapter 7, at zero pipette potentials inward channels were recorded. In one of these experiments the mean amplitude of the channels was 1.4pA, open time was 2.0±1.6ms (mean ± SD, n=937) and the close time was 394±960ms (mean ± SD, n=937). Hence the percentage open time of these channels was 0.5%. This egg was

Figure 3.10

A. Current voltage relations of two types of channels recorded in a cell attached patch on an egg bathed in Ca10K5. Pipette filled with Sr60/TEA/CsCl. The conductance of one group of channels was 13pS (marked "calcium") and that of the other was 46pS (marked "chloride"). Ba. Consecutive pen trace records of inward currents observed in a cell attached patch on an egg bathed in Ca10K5. The patch pipette was filled with Ca10K5 diluted to 90%. The corresponding open and closed time distributions are shown in Ca and Cb respectively. Oscilloscope traces of these channel openings are also shown as insets on these distributions. Bb. Consecutive pen trace records of inward currents observed in a cell attached patch on an egg also bathed in Ca10K5 but the patch pipette was filled with normal solution containing 5mM EGTA, but no calcium. The open and closed time distributions of these channels are shown in Da and Db respectively. An oscilloscope trace obtained during the recording, is shown as an inset on the open time distribution (Da). The traces shown as insets in C and D were filtered at 1000Hz, (-3dB) and 350Hz, (-3dB) respectively. All distributions (C and D) were plotted, after filtering the records at 1000Hz, (-3dB).


A
Outward current
(pA)
1.0
Chloride
H40 H30 H20 H10 0 D10 D20 D30 D40 D50
Transpatch potential
(mV)
Calcium
1.0
2.0
Inward current
(pA)
3.0
Single channel
amplitude
B a
c
c
8pA
20ms
b
c
c
8pA
100ms


C a
192
128
64
0
c
2pA
40ms
0 12 24
b
768
512
256
0
c
2pA
160ms
0 1.9 3.8 5.7
Number of observations
D a
1536
1024
512
0
c
8pA
100ms
0 20 40 60
Open time (ms)
b
768
512
256
0
0 0.1 0.2 0.3
Closed time (sec)

bathed in Ca10K5, and the patch pipette was filled with the same solution diluted to 90%. Therefore these inward channels could have been either potassium, chloride, calcium or sodium. In any case the transpatch potential was more negative than the equilibrium potential for the ion causing the observed current flow. (Since the pipette potential was zero, the transpatch potential was assumed to be equal to the resting potential of the egg). These channels are shown as two consecutive pen traces in Fig. 3.10Ba and their open and closed time distributions in Fig. 3.10Ca and 3.10Cb respectively (oscilloscope traces of these channel openings are shown as insets on these distributions).

In the second of these recordings at a zero pipette potential, the mean open and closed times were 3.1±4.4 (mean ± SD, n=3024) and 17.1±22.4 (mean ± SD, n=3024) respectively. Therefore the percentage open time was 15.55%. The amplitude of the inward channels observed, was 3.4pA. These are shown as two consecutive pen trace records in Fig. 3.10Bb, the open and closed time distributions of which are illustrated in Fig. 3.10Da and 3.10Db respectively (an oscilloscope trace is also illustrated as an inset in the open time distribution - Fig. 3.10Da). These channels were recorded from a cell attached patch on an egg bathed in Ca10K5, the pipette was filled with normal solution containing 5mM EGTA, but no calcium. Hence these channels could only possibly be potassium or chloride

or sodium.

Finally in the third such recording (not illustrated) the amplitude of the inward currents was 1.2pA, the bathing solution was Sr1OK5 and the pipette was filled with Ca1OK5 diluted to 90%. Hence in this case the channels could have been either potassium, chloride, calcium or sodium. Since in these experiments the transpatch potential is not varied, no indication of the reversal potential was obtained for these channels. Hence it is not possible to conclude what type of channels these were.

3.8 Discussion

These experiments give a preliminary indication of the types of channels present in the mouse egg membrane. There appear to be at least four types:-

a) a large potassium channel, the conductance of which is about 120pS. Potassium flows out of the egg via this channel and shows rectification (delayed or outward rectification) with increasing depolarizations of the patch.

b) a large potassium channel, the conductance of which is about 90pS. Potassium flows into the egg via this channel, and this channel also shows rectification, but with increasing hyperpolarizations of the patch (anomalous or inward rectification).

c) a possible chloride channel with a conductance of about 50pS. These channels were observed in

experiments in which the patch pipettes were filled with Sr60/TEA/CsCl, and are therefore unlikely to be potassium channels. The possibility of there being a potassium channel with a similar conductance was not ruled out in all experiments. But knowing the reversal potential for potassium responses, from past published work (Igusa et al., 1983), it seemed unlikely that these were potassium channels.

d) a group of channels interpreted as being calcium, were always inward, with a small conductance (7-20pS). Analysis of these calcium channels was difficult because of their small size and the "contaminating presence" of potassium and chloride channels.

Five current voltage relations on potassium channels showed both an inward and outward rectification (e.g. Fig.3.3B). Such plots were divided into three phases, as described in 3.3. The H phase corresponded to the inward or anomalous rectifying phase, and the D phase to the outward or delayed rectifying phase. The two potentials splitting the three phases, were "judged by eye" and the conductances quoted for each phase are the maximum slope conductances. It is unlikely that one type of potassium channel rectifies in both the outward and inward direction. Therefore such outward and inward potassium currents, have been interpreted as flowing through two types of channels, described as being outward and inward rectifiers respectively (described in a and b above).

Examples of inward channel current records, in which one channel was open for a large proportion of the time, but with no double openings, are shown in Fig.3.2A (eg transpatch potentials of H40-H70). Such records indicate that all the openings are of the same channel. It is possible to calculate the probability P of there being N channels in a given recording using the following equation derived by Colquhoun and Hawkes (Sakmann and Neher, 1983).

$$P=\pi^{n} \quad \text{where } \pi = \frac{1}{1+\left(\frac{N-1}{N}\right)\frac{Mo}{Ms}}$$

where Mo = mean open time

Ms = mean closed time

n = number of openings

For recordings in which Ms>>Mo, calculations indicate that the probability (P) of there being two channels (ie N=2), may be as high as 0.80. For example, in one observation the mean open (Mo) and the mean closed (Ms) time was 3.0 and 458.7ms respectively, and the number of openings (n) was 67.

Outward potassium currents

During voltage clamp experiments (on zona-free mouse eggs), by Okamoto et al (1977) a depolarization of the membrane over +50mV from the holding level of -75mV, elicited an "outward surge current". This current showed outward rectification. An inward flux

of chloride was not responsible for this outward surge current because it remained unchanged in chloride free media. Okamoto et al (1977) postulated that the outward surge current was caused by a delayed rectification due to potassium channels. The delayed rectification is suppressed by external TEA ions in Nodes of Ranvier of frog myelinated axons (Hille, 1967). But in the mouse egg, the replacement of external sodium ions by TEA on an equimolar basis, or the injection of caesium into the egg had no effect on the outward surge current.

Miyazaki and Igusa (1982) obtained current voltage relations for zona-free hamster eggs before insemination. Similar curves were plotted at the peak of hyperpolarizing responses elicited after fertilization of these eggs (1.21). Subtraction of these two curves gave current voltage relations for the calcium activated potassium conductance, assuming that the leakage conductance was unaltered. Such current voltage relations for the calcium activated potassium conductance also showed an outward rectification.

Igusa and Miyazaki (1983) and Georgiou et al (1983) have described a calcium activated potassium conductance in hamster eggs. There is a "less sensitive" calcium activated potassium conductance in mouse eggs, since injections of calcium gave smaller hyperpolarizing responses in mouse eggs, than those observed in hamster eggs (see 1.21). Therefore an investigation of calcium activated potassium channels,

would probably be best performed using hamster eggs. The outward potassium currents described in this chapter may be calcium activated potassium currents, which could be further studied using excised patches of membrane, with the intracellular membrane surface exposed to different concentrations of calcium (Barrett, Magleby & Pallotta, 1982).

In endocrine cells, calcium activated potassium channels are opened by an increase in cytosolic calcium. The latter is brought about by a calcium influx through voltage gated calcium channels which are opened by depolarization and closed by hyperpolarization. As the membrane hyperpolarizes due to potassium efflux via the calcium activated potassium channels, further calcium influx is prevented. In typical endocrine cells such as chromaffin and pancreatic beta-cells, as well as nerve endings, calcium influx is needed for excitation secretion coupling (Baker, Hodgkin & Ridgway, 1971; Reuter, 1983). A similar "secretion" of cortical granules (i.e. the cortical reaction) is observed after fertilization.

In the study by Barrett et al (1982), calcium activated potassium channels were observed to enter a conductance state in which the current through a single channel was about 40% of the amplitude of the "normal". It may therefore be argued that the channels denoted "C" in Fig.3.7A are such a sub-conductance state of the

larger potassium channel ("K"). But in the record shown in Fig.3.7A these small channels were open for 52% of the total recording time, and the larger potassium channels were open for 16% of the total recording time. By comparison, Barrett et al (1982) observed the openings of reduced amplitude for 0.1% of the total channel open time. In the present study these smaller channels ("C") have been interpreted as being chloride channels.

Inward potassium currents

Anomalous rectification was originally observed in the frog muscle membrane by Katz, but has more recently been studied by Adrian and Freygang (1962). It has also been observed in the egg membrane of the tunicate (Ohmori, 1978) and the starfish (Okamoto et al, 1976b). The potassium inward current through the rectifier shows fast activation and later a slow inactivation, in response to a hyperpolarizing step change of membrane potential. Slow inactivation of the inward potassium current has also been observed in frog skeletal muscle, where it has been studied quantitatively by the voltage clamp technique (Almers, 1972). The slow inactivation of the potassium inward current in the tunicate egg has been studied by Ohmori (1978). He noted that the inactivation followed first order kinetics and that the steady state inward current through the anomalous rectifier in the tunicate egg cell membrane, showed marked fluctuations when the

membrane was hyperpolarized to about -150mV. This may be an explanation for the fluctuations in the membrane potential observed when mouse and hamster eggs were current clamped at large negative potentials (in excess of -80mV) in this study.

In nine cell attached patches (eggs bathed in Ca4K5 + La2 and pipette filled with K155 + EGTA) the mean number of inward potassium channels observed was 1.22 (two channels in two patches and one in each of the rest).

In other preparations the conductance of the anomalous potassium rectifier channel is found to be approximately proportional to the square root of the external potassium concentration (Hagiwara & Takahashi, 1974; Ohmori, 1978, 1980). In the experiments described in 3.3, the conductance of the inward potassium channel was 90±31pS (n=6) when the pipette was filled with K155 + EGTA and values of 125, 64 and 80pS, were obtained when the patch was filled with K155 + EGTA diluted to 80%. This discrepancy may be explained by one or more of the following:-

a) the pipette filling solution in the above experiments was an "unphysiological extracellular medium", since it contained 155mM potassium and 10^{-9}M calcium. Furthermore, sodium which is a time dependent blocker of this channel is not present (Ohmori, 1978, 1980).

b) these channels are observed to have a Q_{10} of 1.5

(Fukushima, 1982). In the present study, the temperature of the bathing solution was 34-35^{o}C, whereas the bath temperature in the study by Fukushima (1982) was 14-15^{o}C.

c) it may be that the currents observed on hyperpolarization are not inward potassium currents, but are in fact outward chloride currents. This last possibility will have to be investigated carefully.

Okamoto et al (1977) observed that during hyperpolarizations below -200mV, from a holding potential level of -50mV, an instantaneous increase in the inward current was evoked in mouse eggs. The I-V relation at the peak of the inward current showed a marked inward going rectification. They suggested that breakdown of the membrane may be responsible for the increase in inward current, because of the large negative potential required to induce the rectification. But since their recordings involved having an intracellular microelectrode inserted into the egg, this may have caused an impalement leak (Chapter 4). The leak may explain the wide range of potentials (+25 to -200mV) over which the current voltage relation was linear. Thus the potential required to induce the "inward rectification" may have been overestimated in the negative direction. Whereas in the present study, the current voltage relation was plotted from amplitudes of single channel currents recorded with patch pipettes, i.e. there was no intracellular electrode, and hence it is believed that

there was no "impalement leak".

The reversal potential of the single anomalous potassium channel current (obtained by extrapolation of its linear current voltage relation) obtained in tunicate eggs (Fukushima, 1982), was always 10-20mV more negative than the reversal potential of the macroscopic current (which is equal to the potassium equilibrium potential $-E_k$). This discrepancy suggests that the single channel current itself also shows inward rectification. Rectification of the single channel conductance can be obtained if the channel has multiple sites with multiple ion occupancy (Hille & Schwartz, 1978). If such is the case in this study, then all the resting potentials estimated in 3.3 will have been over-estimated by 10-20mV.

The presence of inward rectifiers in eggs of starfish and tunicates, has been rationalised on the basis of the long (in some cases several minutes) depolarizing fertilization potentials observed after sperm entry into the egg (see 1.16 and 1.18 respectively). The function of inward rectifiers in skeletal muscle is more speculative. Possible roles in this preparation include (i) a device to clamp the membrane potential near E_k, in the face of a very active electrogenic sodium pump, tending to hyperpolarize the membrane and (ii) a pathway for potassium re-entry from potassium loaded transverse tubules after an action potential. It remains to be

investigated, if any similar role is applicable to inward rectifying channels in mammalian eggs.

Chloride channels

The chloride conductance is activated at fertilization in R.pipiens eggs, serving to depolarize the eggs during the fertilization potential (see 1.19). But in addition to this the immature R.pipiens oocyte membrane contains a voltage activated chloride conductance, which disappears during maturation (Schlichter, 1983). This conductance is not activated at potentials more negative than 0mV, and is sensitive to SITS. Whereas the chloride conductance activated at fertilization is SITS insensitive, but is activated at potentials as negative as -25mV (Jaffe & Schlichter, 1985). Both these conductances increase with depolarization, thereby increasing the rate of rise of the fertilization potential and, hence the rate of establishment of the fast electrical block to polyspermy.

Current voltage plots of "possible" chloride channels in this study were linear (Figs.3.7Bb and 3.7Cb).

Four chloride conductances have been described in the Xenopus oocyte:-

a) activated at fertilization (Grey et al., 1982)

b) stimulated by acetylcholine (Kusano et al., 1982)

c) present at the resting potential of the egg (Robinson , 1979) and

d) a rapidly inactivating chloride conductance stimulated by calcium (Miledi, 1982).

In amphibians the equilibrium potential of chloride (E_{Cl}) is positive [in study by Jaffe & Schlichter, (1985) E_{Cl} = +18mV] and hence at the resting potential (about -30mV), chloride will flow out of the egg into the surrounding medium thereby causing a depolarization of the egg.

Powers and Tupper (1975) have reported a value for E_{Cl} of -15mV, in mouse eggs. This value was obtained by measuring the distribution of ^{36}Cl in the bathing medium and the cytosol, after a period of incubation in ^{36}Cl containing solutions. Their results indicated an internal exchangeable chloride concentration of 67$\pm$3mM. This result, and hence their estimate of E_{Cl} may be in error, because in their experiments no correction was made for the distribution of chloride in the perivitelline space or the zona pellucida.

In mammalian heart muscle, measurement of intracellular chloride activity, with chloride sensitive microelectrodes indicated values for E_{Cl} which were about 35mV more positive than the resting potential (E_{Cl} = -42.9mV and resting potential of sheep cardiac purkinje fibres = -78.7$\pm$0.9mV; Vaughan-Jones, 1979). Assuming that E_{Cl} in mammalian eggs is about -40mV, then the resting potential of the eggs from which the results in Fig.3.7B and 3.7C were obtained, was -81mV and -59mV respectively.

Calcium channels

Single calcium channel currents have been recorded directly using the giga-ohm seal patch clamp technique on intact cells, e.g. neonatal rat heart cells (Reuter, Stevens, Tsien & Yellen, 1982), bovine chromaffin cells (Fenwick, Marty & Neher, 1982b) and clonal rat pituitary cells - GH_3 (Hagiwara & Ohmori, 1983). Even with high external concentrations of barium, the observed amplitude of the unitary current was smaller than 1pA, except for the calcium channel of heart cells where the unitary current reaches about 2pA. In the present study the mean amplitude of single calcium channels was 1.1pA.

Based on the results of this study it is not possible to rule out the presence of sodium channels in mouse and hamster egg membranes. In order to keep their detection to a minimum, sodium free pipette filling solutions were employed in most experiments. Of the various types of pipette filling solutions used, in only Ca10K5 was there any sodium present. In future experiments to study calcium channels it may be advisable to add sodium channel blockers, e.g. TTX (tetrodotoxin), to the pipette filling solution, even in the absence of sodium, since the calcium permeability of sodium channels is generally not known. This is in case sodium channels are present in hamster and mouse egg membranes.

During experiments to study calcium channels,

serious problems were encountered due to insufficient elimination of potassium or chloride or other non specific currents. Currents due to potassium channels and other non specific channels would be expected to become prominent at large positive transpatch potentials, at which calcium current fluctuations become small.

The amplitude of single calcium channel currents decreases with depolarization of the patch (negative pipette potentials), as expected for an apparent reversal potential for whole cell calcium currents at about +50 to +60mV in heart cells (Lee & Tsien , 1982) and chromaffin cells (Fenwick et al, 1982b). Conductances of calcium currents in 40mM calcium of about 7pS have been observed in Helix (Lux & Nagy, 1981; Brown, Camerer, Kunze & Lux, 1982). Similar conductances have been obtained for barium currents in 100mM barium observed in pituitary cells (Hagiwara & Ohmori, 1982) and for those in isotonic barium noted in chromaffin cells (Fenwick et al., 1982b). Larger conductances (about 25pS) have been reported for cultured heart myocytes in isotonic barium (Reuter et al ., 1982). All these values are higher than previous values obtained from fluctuation analysis of Helix calcium and barium currents (Akaike, Fishman, Lee, Moore & Brown, 1978; Krishtal, Pidoplichko & Shakhovalov, 1981). Three values for the conductance of calcium channels were obtained in this study - 7.5,

13 and 20pS.

There have been two reports of reversal of current through the calcium channel, one in heart cells (Lee & Tsien, 1982) and the other in chromaffin cells (Fenwick et al., 1982b). In these two examples the reversal potential is observed at potentials more negative than the Nernst potential for calcium. Therefore it is likely that the outward current is not carried by calcium, but probably by the main intracellular cation, i.e. potassium or caesium.

In the studies by Fenwick et al. (1982a) and Hagiwara and Ohmori (1982), the pipette input resistances after the formation of a seal between the pipette and the membrane (chromaffin cells and rat anterior pituitary tumour cells respectively) ranged between 10-50GΩ and 10-100GΩ respectively. In the present study such seal resistances (between pipette and egg membrane) lay in the range 200MΩ - 2GΩ although on one occasion a 30GΩ seal resistance was obtained. It is likely that these lower seal resistances obtained with the egg preparation, are due to the surface microvilli. A consequence of this is that the background noise was too large, to allow analysis of opening and closing behaviour. Voltage clamp experiments by Okamoto et al (1977) indicated that the selectivity ratios for the inward currents, elicited by hyperpolarizing pulses (the current flows through calcium channels, which open in synchrony with anode break spikes) were Ca: Sr: Ba: = 1.0: 1.4: 0.7.

Hence in an effort to increase the amplitude of inward currents (on the assumption that the mean open time for the calcium channel is no different with different ion carriers) observed through calcium channels, calcium was replaced by strontium in the bathing and pipette filling solutions.

Byerly and Hagiwara (1982) reported that calcium currents subside after 20 to 40 minutes in dialysed snail neurones. Fenwick et al (1982b) similarly observed that calcium currents could be recorded no longer than 10-20 minutes before an irreversible decline occurred. The current decreased to about 30% of its initial value after about 20 minutes. Fenwick et al (1982b) suggest that the decrease in current is due to the decrease in the number of channels available for activation, rather than a decrease in the unitary conductance or individual opening probability. They observed that this so called rundown was accelerated in three ways:-

1. it was faster with higher calcium concentrations in the pipette.
2. it was faster if larger calcium currents were induced by voltage pulses and
3. a very fast rundown occurred following the formation of an isolated membrane patch (inside out or outside out). Single barium currents were observed for no more than 30 seconds to 2 minutes in isolated patches, after which they were totally inactivated.

Byerly and Hagiwara (1982) suggested that activation of calcium channels needs an intracellular substance. Fenwick et al (1982b) observed that in chromaffin cells which appeared granular ("metabolic state of the cells was questionable"), calcium currents were small or absent, whereas sodium and potassium currents still appeared normal. Such cells may have lost an intracellular substance that activates calcium channels. In some experiments in the present study, the eggs appeared granular, the percentage of which increased with age (post-HCG injections). Such a "rundown" may be occurring in these eggs, simply after removal of the zona pellucida.

CHAPTER 4 A PRELIMINARY INVESTIGATION OF THE RESTING POTENTIAL, AND FURTHER EXPERIMENTS WITH CELL ATTACHED PATCHES

4.1 Impalements of zona-free hamster eggs

4.2 Mean membrane potentials and mean input resistances of zona-free hamster eggs bathed in different solutions

4.3 Techniques employed to enhance the sealing of the egg membrane to the microelectrode tip

4.4 The effect of temperature on the membrane potential and the input resistance recorded in zona-free mouse eggs

4.5 Recording channel currents at the same time as passing hyperpolarizing pulses through an intracellular microelectrode

4.6 Recording channel currents at the same time as passing depolarizing pulses through an intracellular microelectrode

4.7 Depolarizing pulses applied across the patch during cell attached patch recording

4.8 Preliminary attempts at measuring whole cell potentials of zona-free hamster eggs

4.9 Discussion

In this chapter are discussed the possible errors introduced in electrical recordings by the impalement of zona-free eggs with intracellular microelectrodes. Then the assumption is made, that it is not possible to overcome these errors using the above technique and other methods of obtaining an indication of the resting potential of these eggs are explored. The latter half of this chapter is almost a continuation of the experiments described in Chapter 3, where the voltage sensitivity of some of the channels in the egg membrane is discussed.

A lot of these experiments are preliminary and give indications rather than firm conclusions of the true resting potential of zona-free hamster or mouse eggs.

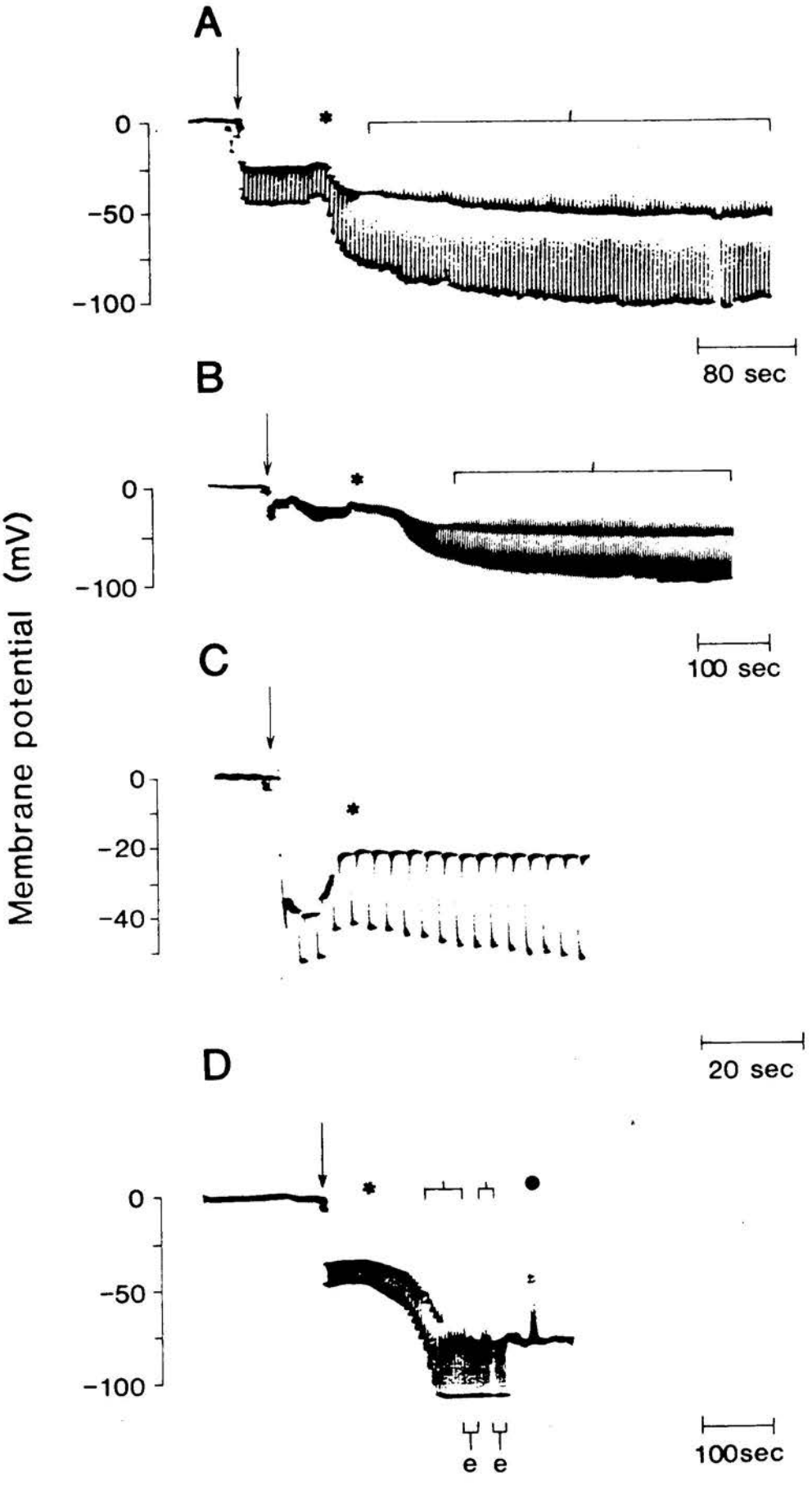
4.1 Impalements of zona-free hamster eggs (Table 4.1)

The membrane potential recorded, within one or two seconds after the impalement was variable in the whole population of eggs. For example, in the pen traces of four impalements shown in Figure 4.1, such measurements of initial potential yielded the following results: -25mV in A, -15mV in B, -36mV in C and -39mV in D (the result in C should be ignored at this stage, for reasons explained below). This variable initial measurement of the membrane potential is indicative of a leak around the microelectrode caused by the impalement (Georgiou et al, 1983).

During impalements hyperpolarizing current pulses

Figure 4.1

Pen trace records of impalements of zona-free hamster eggs, (with intracellular microelectrodes) bathed in A Ca2K25, B Ca4K5, C normal solution containing 42mM calcium chloride and 80mM sodium chloride and D Ca4K5. Arrows indicate the time when the electrode, which was resting on the egg membrane, was oscillated by applying increasing negative capacity - technique used for impaling these eggs (see Chapter 2). Asterisks refer to the start of the "sealing process" (see text), brackets denote the period during which anode break responses were observed and the filled circle indicates when depolarizing pulses passed through the electrode elicited "electrically evoked action potentials". Hyperpolarizing pulses elicited electrotonic potentials during the periods marked "e" in D, when the egg had a high membrane potential. Towards the end of the trace shown in D, the downward deflections were truncated by the limited travel of the pen. All the current pulses were 0.25nA and 1sec. In A and D they were 0.25Hz, and in B and C they were 0.30Hz.


A
0
-50
-100
80 sec
B
0
-100
100 sec
C
0
-20
-40
20 sec
D
0
-50
-100
e e
100sec
Membrane potential (mV)

were passed through the microelectrode to measure the input resistance of the egg (2.3a). These current pulses caused electrotonic potentials which are indicated by downward deflections on the pen traces. In general the membrane potential increased (became more hyperpolarized) and simultaneously the input resistance of the egg increased, as measured by the increase in the amplitude of electrotonic potentials (since current pulses were of constant amplitude, this indicated an increase in input resistance). The beginning of this increase in the input resistance is marked by asterisks in Figure 4.1. Such increases in the input resistances are interpreted as being due to the "sealing" of the egg membrane around the microelectrode (Hagiwara & Jaffe, 1979; Petersen, 1980). On occasions it was necessary to wait for up to 30 minutes for the membrane potential and the input resistance to reach steady maximum values.

Hyperpolarizing current pulses evoked anode break responses in eggs provided that the membrane potential reached about -80mV during the course of the pulse as observed by Miyazaki & Igusa (1982). Such responses are due to the opening of voltage sensitive calcium channels which are inactivated at potentials more positive than about -60mV (Okamoto et al., 1977). Such anode break responses were observed during the periods indicated by the brackets in Figure 4.1 (A,B and D). The threshold of anode break responses varied between

Table 4.1

Details of five zona-free hamster eggs which had high membrane potentials, when impaled with intracellular microelectrodes. Chapters refer to the chapter in which of these eggs was discussed. The egg bathed in Ca10K5 was not fertilized and is not therefore discussed in the text. Attempts were made to fertilize all five eggs with hamster sperm except one in which mouse sperm were used (indicated in the Table). All five of these cells were electrically excitable, although threshold measurements were not made in two of them (indicated by asterisks).

Bathing Solution	Chapter	Membrane potential(mV)	Input resistance(MΩ)	Threshold of electrically evoked action potential(mV)	Spike peak of electrically evoked action potential(mV)	Fertilization attempted	Fertilization successful
Ca4K5	5	-100	200	-60	-19	✓	✓
Ca4K5	5	-70		*		✓ mouse sperm	✓
Ca4K5	5	-75	250	-64	-44	✓	✓
Ba10K25	6	-61	340	-56	0	✓	✓
Ca10K5		-65	300	*		✓	X

TABLE 4.1

approximately -45mV and -65mV, depending on the bathing solution (there was also a variability in the eggs).

If the membrane potential increased to a potential more negative than the threshold for anode break responses, then hyperpolarizing pulses again only elicited electrotonic potentials (Figure 2.4 and 2.5.), such as in the two regions marked by "e" below the trace in Figure 4.1D. At such potentials the passage of depolarizing pulses through the electrode elicited action potentials (Figures 2.4 and 2.5, marked by filled circle in Figure 4.1D).

Throughout this study in only five hamster eggs did the membrane potential reach a value more negative than the threshold for the anode break (which was the same as that for the action potential in any given egg). All five of these eggs were electrically excitable, i.e. action potentials could be evoked on the passage of depolarizing pulses. This is out of a total of 247 experiments, in which each egg was impaled only once. All these impalements are scored in Chapters 5, 6 and 7. Details of these five eggs are given in Table 4.1. Such membrane potentials which are more negative than the threshold for the action potential (called the electrically evoked action potential in the text), have been referred to as being high membrane potentials, and those more depolarized than the threshold as being low membrane potentials. It is possible therefore that in the majority of experiments (98% with the above mentioned figures,

although percentage is probably much closer to 100%, since in several hundred other experiments done by C.R. House and P. Georgiou, no high membrane potentials were observed) there exists a leak pathway at the electrode tip, which depolarizes the membrane potentials recorded in hamster eggs (or mouse) (Georgiou et al., 1983 and Ph.D. Thesis, P. Georgiou, University of Edinburgh, 1985).

Throughout this study many experiments were performed (Chapters 5,6,7 and Appendix A) in which low membrane potential eggs were current clamped at various potentials. These potentials were referred to as being high or low current clamp potentials (to distinguish from "high" and "low" membrane potentials in which no constant current was passed through the electrode), if the potential was below (more negative) or above (more positive) the threshold of the electrically evoked action potential respectively.

The reason for current clamping the eggs at high current clamp potentials was to try to observe the electrical events which might occur during fertilization of hamster eggs, if indeed the resting potential of the egg was high.

Occasionally after impalements of some hamster eggs a transient hyperpolarization followed by a depolarization was observed, accompanied by an increase in resistance (it would be expected that a decrease in resistance would accompany a depolarization, if the

leak pathway conductance around the electrode was increasing), as illustrated in Figure 4.1C. Such a response usually lasting no more than 30 seconds, was followed by an increase in the membrane potential, accompanied by an increase in input resistance. This type of response occurred more frequently if the egg was bathed in a solution containing a high concentration of calcium (or strontium) than that present in normal solution. These responses have been attributed to a calcium activated potassium conductance which is present in mouse and hamster egg membranes. The leakage around the microelectrode tip, produced during impalement, provides a low resistance shunt for movement of calcium from the bathing medium into the cytosol of the egg, which is sufficient to activate the potassium conductance. This then causes a hyperpolarization (if the membrane potential of the egg is low), because the equilibrium potential for potassium is about -80mV (Chapter 5; Miyazaki & Igusa, 1982, Georgiou et al., 1983).

4.2 Mean membrane potentials and mean input resistances of zona-free hamster eggs bathed in different solutions

Sufficient time (10-30 minutes) was allowed to elapse, for the membrane to seal as much as possible around the electrode. Then the steady membrane potential and input resistance values were noted. In Figure 4.1 the final steady values of membrane potential were -51mV (A), -48mV (B), -30mV (C - not

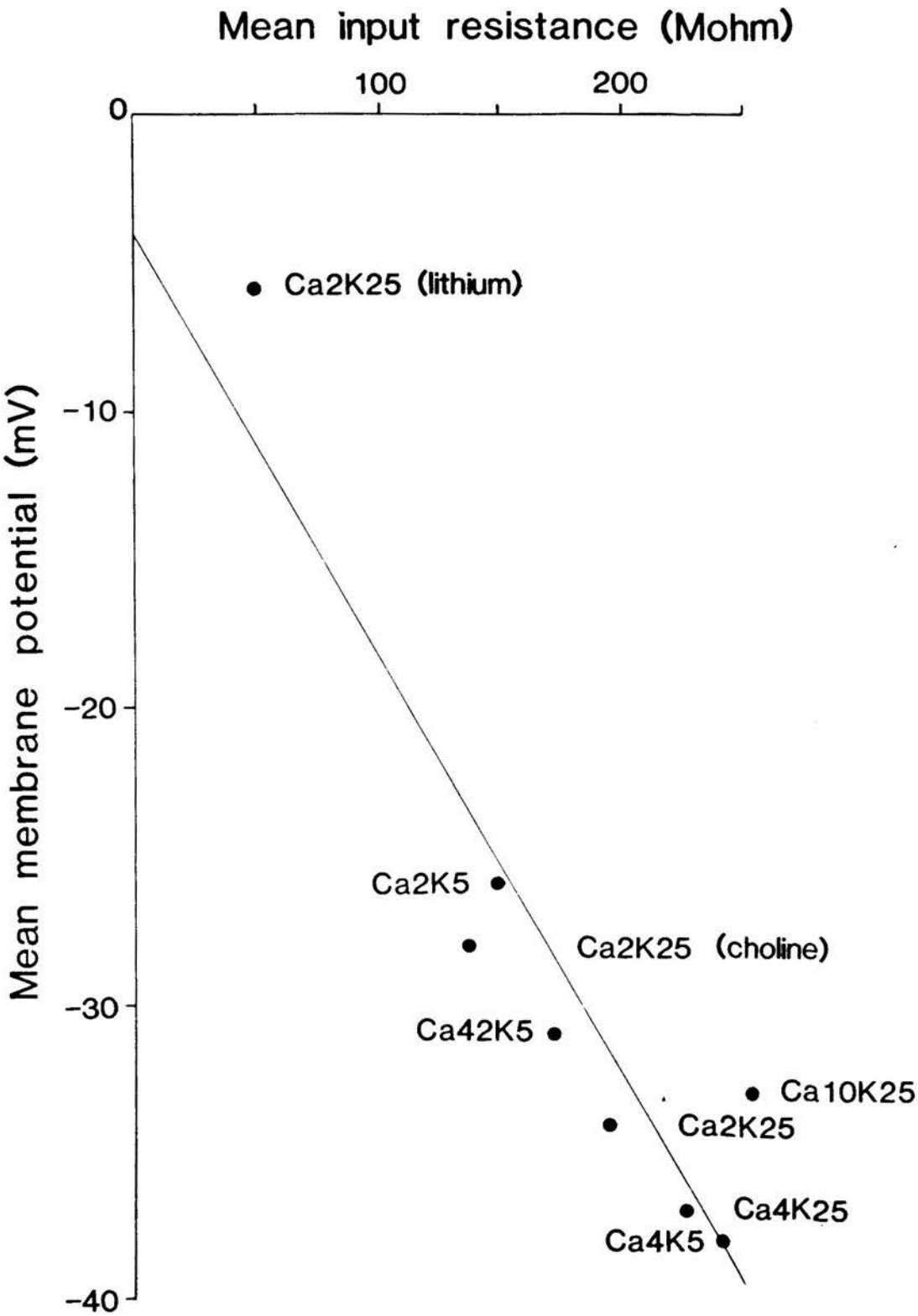
shown) and -75mV (D) and the final steady value of the input resistance were 225MΩ (A and B), 170MΩ (C) and 250MΩ (D). The impalement shown in Figure 4.1D is also noted in Table 4.1.

In any given egg a larger input resistance was associated with a larger membrane potential. The mean ± SD was calculated for the final steady membrane potential and input resistance for the whole population of eggs impaled in any given solution. These are given in Chapter 5. But the mean membrane potentials in normal solution (Ca2K5), normal solution containing 4mM calcium (Ca4K5) and in high potassium solution (Ca2K25) were -26±8mV (n=31), -38±14mV (n=56) and -34±8mV (n=46) respectively. Similarly the mean input resistances in Ca2K5, Ca4K5 and Ca2K25 were 149±69MΩ (n=31), 241±96MΩ (n=55) and 195±56MΩ (n=46) respectively. In general large input resistance eggs have large membrane potentials (as indicated by negative correlation coefficients of input resistance versus membrane potential). There was a significant correlation between the input resistance and membrane potential, of the population of eggs in any given solution. The correlation coefficients were -0.60, ($p<0.001$, n=46) in Ca2K25; -0.61, ($p<0.001$, n=31) in Ca2K5 and -0.31, ($p<0.05$, n=57) in Ca4K5.

When the mean values of the membrane potentials recorded in different solutions were plotted against the respective mean input resistances (Figure 4.2) a

Figure 4.2

A graph of the mean membrane potentials plotted against the mean input resistances of zona-free hamster eggs bathed in different solutions. The membrane potentials and input resistances were measured with intracellular microelectrodes. The compositions of each of the solutions and the actual mean values are given in Chapter 5.


Mean input resistance (Mohm)
0
100
200
Mean membrane potential (mV)
-10
-20
-30
-40
Ca2K25 (lithium)
Ca2K5
Ca2K25 (choline)
Ca42K5
Ca10K25
Ca2K25
Ca4K25
Ca4K5

good correlation was observed ($r=-0.93$, $p<0.001$, $n=8$) This plot also indicated that larger input resistances were associated with larger membrane potentials.

4.3 Techniques employed to enhance the sealing of the egg membrane to the microelectrode tip

Chambers and de Armendi (1979) found that the application of hyperpolarizing current improved the sealing process at the microelectrode tip as reported for Aplysia neurones (personal communication by E. Mayeri, cited by Brown & Flaming, 1977). During the intracellular recording experiments described in this study hyperpolarizing current pulses were applied to the egg during and after impalement.

Whilst performing the experiments on any given day it was considered that an increased calcium concentration in the bathing solution to 4mM resulted in better impalements of eggs (as indicated by higher membrane potentials and input resistances). The membrane potentials and input resistances observed in Ca4K5 were significantly higher than those in Ca2K5 ($p<0.01$; Fisher Behrens Test) but no significant differences were found between the membrane potentials and input resistances recorded in eggs bathed in Ca2K25 and Ca4K25 ($p>0.05$, Fisher Behrens test).

4.4 The effect of temperature on the membrane potential and the input resistance recorded in zona-free mouse eggs

In eggs bathed in normal solution at room temperature (20-22°C), the input resistances as measured by intracellular electrodes were as high as 700MΩ (see Mean values above). When the solution bathing an already impaled egg was warmed to 34°C the input resistance fell by 53±18% (n=17; calculated as the difference divided by the initial input resistance, multiplied by 100). The membrane potential simultaneously depolarized by up to 8mV in 14 eggs, hyperpolarized in two eggs (by 3 and 4mV) and no change was observed in one egg.

The decrease in the input resistance is probably a reflection of:-

a) the change in the seal resistance between the electrode tip and the egg membrane and

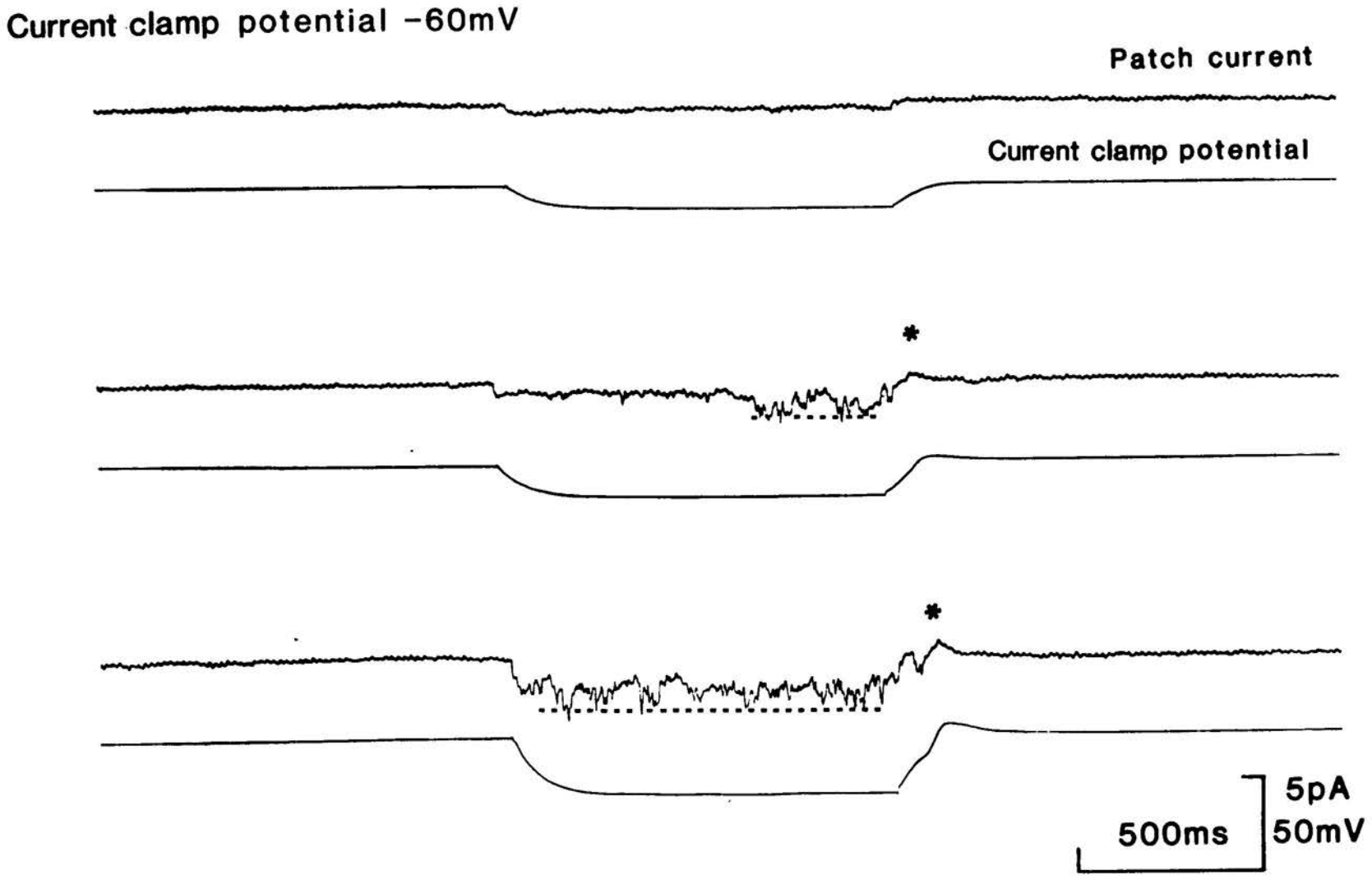
b) an increased channel activity in the egg membrane, as observed in Figure 3.1.

4.5 Recording channel currents at the same time as passing hyperpolarizing pulses through an intracellular microelectrode

It was possible to form a cell attached patch on an egg (see Chapter 3) and then subsequently impale it with an intracellular microelectrode. Hyperpolarizing pulses of variable magnitude could then be passed through the intracellular electrode after it had attained a steady membrane potential and input resistance. One such result of an experiment is

Figure 4.3

Simultaneous voltage and patch current records from an egg bathed in normal solution. The patch pipette was filled with Sr60/TEA/CsCl. The egg was current clamped at -60mV, and three different sizes of hyperpolarizing pulses were passed through the intracellular microelectrode. The current pulses hyperpolarized the membrane to -72mV (top pair), -78mV (middle pair) and -83mV (bottom pair). The downward deflection on the patch current trace indicates an inward current. Note the inward currents in synchrony with the anode break spikes. Asterisks refer to outward currents recorded by the patch pipette at the same time as anode break spikes were recorded by the intracellular microelectrode.


Current clamp potential -60mV
Patch current
Current clamp potential
*
*
5pA
500ms
50mV

illustrated in Figure 4.3, in which there are three pairs of pen traces. The top one of each pair being the current recorded by the patch electrode, and the bottom of each pair is the potential recorded by the intracellular microelectrode. In this experiment the egg was current clamped at -60mV, and hyperpolarizing pulses of three different sizes were passed through it. When the hyperpolarizing pulse was too small to elicit an anode break, an electrotonic potential was recorded by the intracellular electrode (top pair of traces in Figure 4.3). At the same time there was a downward shift in the patch current trace, which was maintained for the duration of the pulse, but no single channel openings were observed. The amplitude of this electrotonic potential was 12mV, the most hyperpolarized potential being -72mV.

When the size of the hyperpolarizing pulse was increased then anode break responses were observed, as in the middle and bottom pair of traces in Figure 4.3. Similarly there was a downward shift of the patch current trace, but also inward channel currents were observed. At the same time as the anode break spike an outward current was detected by the patch pipette, denoted by asterisks in Figure 4.3. The rising phase of the anode break is caused by an inward movement of calcium ions, across the egg membrane (except that portion of the membrane which is enclosed by the patch pipette), which in turn caused an outward capacitative current across the patch, seen as an upward deflection

on the patch current trace.

The downward shift of the patch current trace for the duration of the pulse has been interpreted as being an inward ionic current across the patch (from the pipette into the egg) caused by a hyperpolarizing pulse passed through the intracellular microelectrode.

The amplitude of the hyperpolarizations produced by the hyperpolarizing current pulse in the middle and bottom pair of traces in Figure 4.3 were 18mV and 23mV respectively (i.e. most hyperpolarized potentials were -78 and -83mV respectively). Therefore the threshold for the anode break response was at some potential between -72mV (top trace) and -78mV (middle trace).

The inward channel currents recorded when the membrane potential of the egg was hyperpolarized to at least -78mV, were not observed at potentials more positive than about -72mV, i.e. the channels were inactivated. These channels cannot be inward potassium currents since the patch pipette contained CsCl and TEA. Also in the middle trace of Figure 4.3 the most hyperpolarized potential during the pulse (i.e. -78mV) was more depolarized than the equilibrium potential for potassium and therefore potassium currents would have been expected to be outward. These channels are either calcium or chloride or most probably a mixture of both (see Chapter 3).

Assuming that the chloride equilibrium potential in mouse eggs is about -60mV (it is likely to be more

positive than this - see 3.8) then chloride movement at -72mV, -78mV or -83mV is going to be outward, and would therefore be seen as inward currents on the patch current trace.

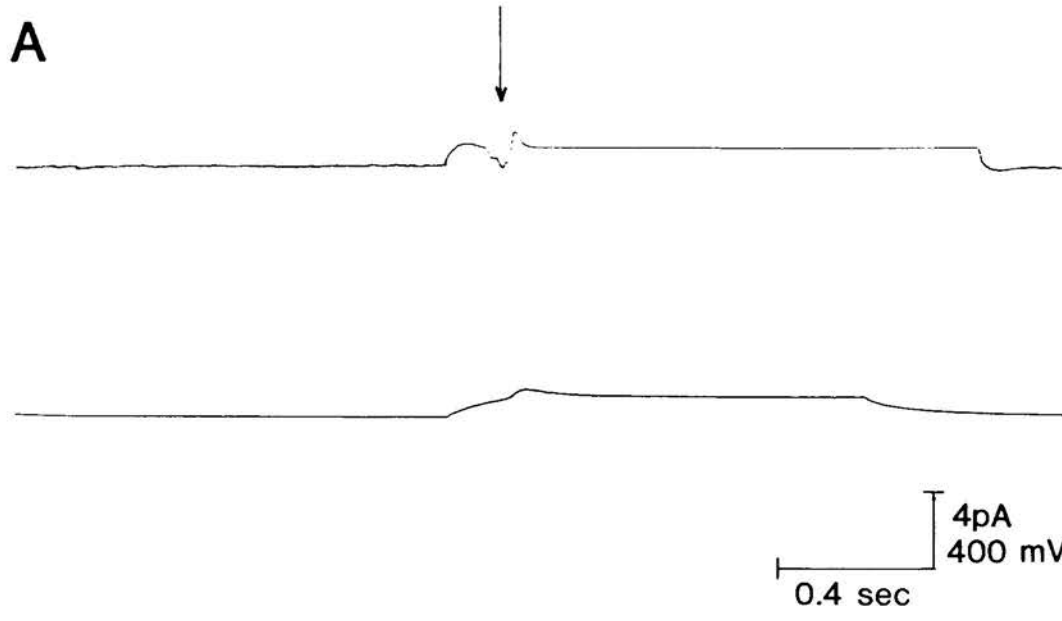
4.6 Recording channel currents at the same time as passing depolarizing pulses through an intracellular microelectrode

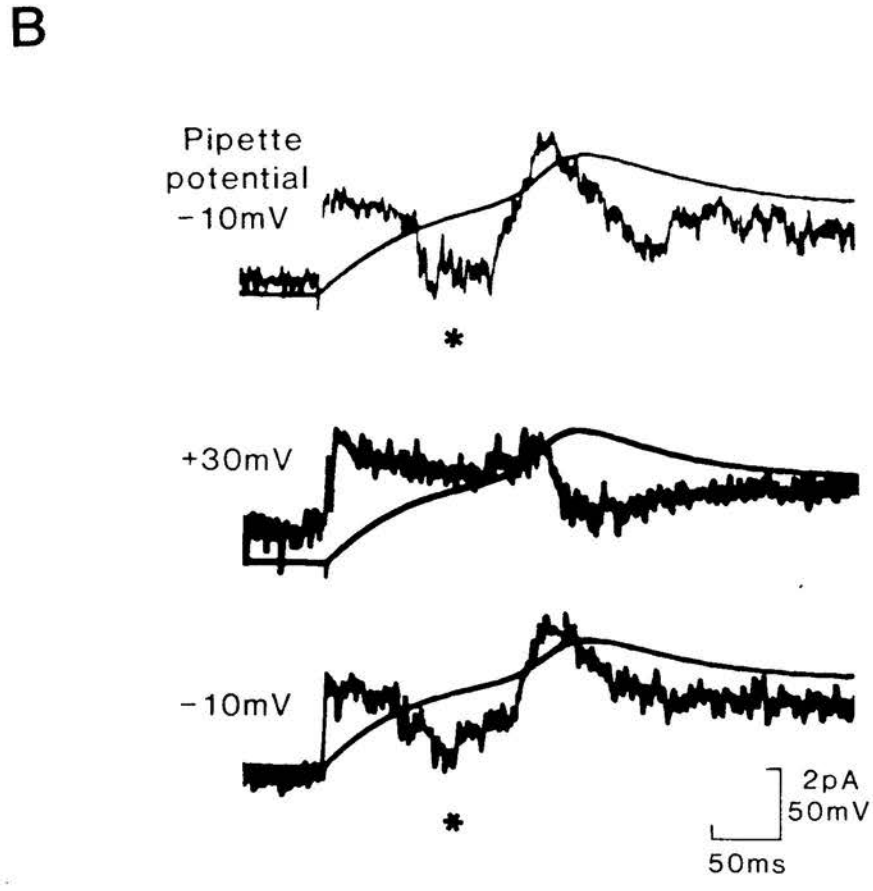
Experiments similar to those described in the previous section, with the exception that depolarizing pulses were passed through the intracellular electrode, are now discussed.

In these experiments the eggs were current clamped at high potentials, the passage then of depolarizing pulses through the intracellular electrode elicited electrically evoked action potentials. One such result is illustrated in the pair of pen traces in Figure 4.4A (this egg was current clamped at -125mV). The bottom trace is the electrically evoked action potential, recorded by the intracellular electrode. The top trace is the patch current recorded simultaneously. The depolarization to the threshold, was seen on the patch current trace as an outward current, interpreted as being the outward ionic current across the patch (into the pipette), due to the depolarizing pulse. As in the anode break spike, the action potential spike is also accompanied by an outward capacitative current (as a consequence of the calcium influx during the spike). Just prior to this outward capacitative current, there

Figure 4.4

Pairs of traces, showing the patch current recorded with a cell attached patch, at the same time as eliciting an action potential, with a depolarizing pulse passed through an intracellular microelectrode. A is a pen trace record and B are oscilloscope pictures. The arrow in A indicates an inward current on the patch current record (top trace). The bottom trace is the voltage record. B - oscilloscope pictures of patch current traces (thicker lines) superimposed on the voltage traces (thinner line), at different pipette potentials. The eggs in A and B were current clamped at -125mV and -120mV respectively. Both eggs were bathed in Sr80K5 and the patch pipettes were filled with Sr80K5 diluted to 90%. A downward deflection on the patch current trace indicates an inward current. Asterisks on the top and bottom pair of traces in B indicate inward currents recorded on the patch current trace.


A
4pA
400 mV
0.4 sec


B
Pipette
potential
-10mV
*
+30mV
-10mV
*
2pA
50mV
50ms

was an inward current, marked by the arrow above the trace in Figure 4.4A. This inward current is due to channel openings in the patch. It is possible, that in this case the transpatch potential is more depolarized than the membrane potential of the rest of the egg, as a consequence of forming a cell attached patch. But this depolarization of the patch membrane is not sufficient to produce a significant channel inactivation.

Figure 4.4B illustrates an experiment in which the patch pipette potential was varied (the three pairs of superimposed traces being recorded in the same egg, current clamped at -120mV), whilst passing a constant depolarizing pulse through the intracellular electrode. In the top pair of traces in Figure 4.4B the pipette potential was -10mV, i.e. the patch was depolarized by 10mV relative to the rest of the egg, and therefore the transpatch potential was 10mV closer to the threshold for the activation of these inward currents (presumably the threshold of the electrically evoked action potential, if these currents are due to an influx of calcium ions). Hence when a depolarizing pulse was passed through the electrode an action potential was elicited and channel opening observed in the patch (marked by an asterisk below the patch current trace).

When the pipette potential was changed to +30mV (as in the middle pair of traces in Fig.4.4B), the patch was hyperpolarized by 30mV relative to the rest

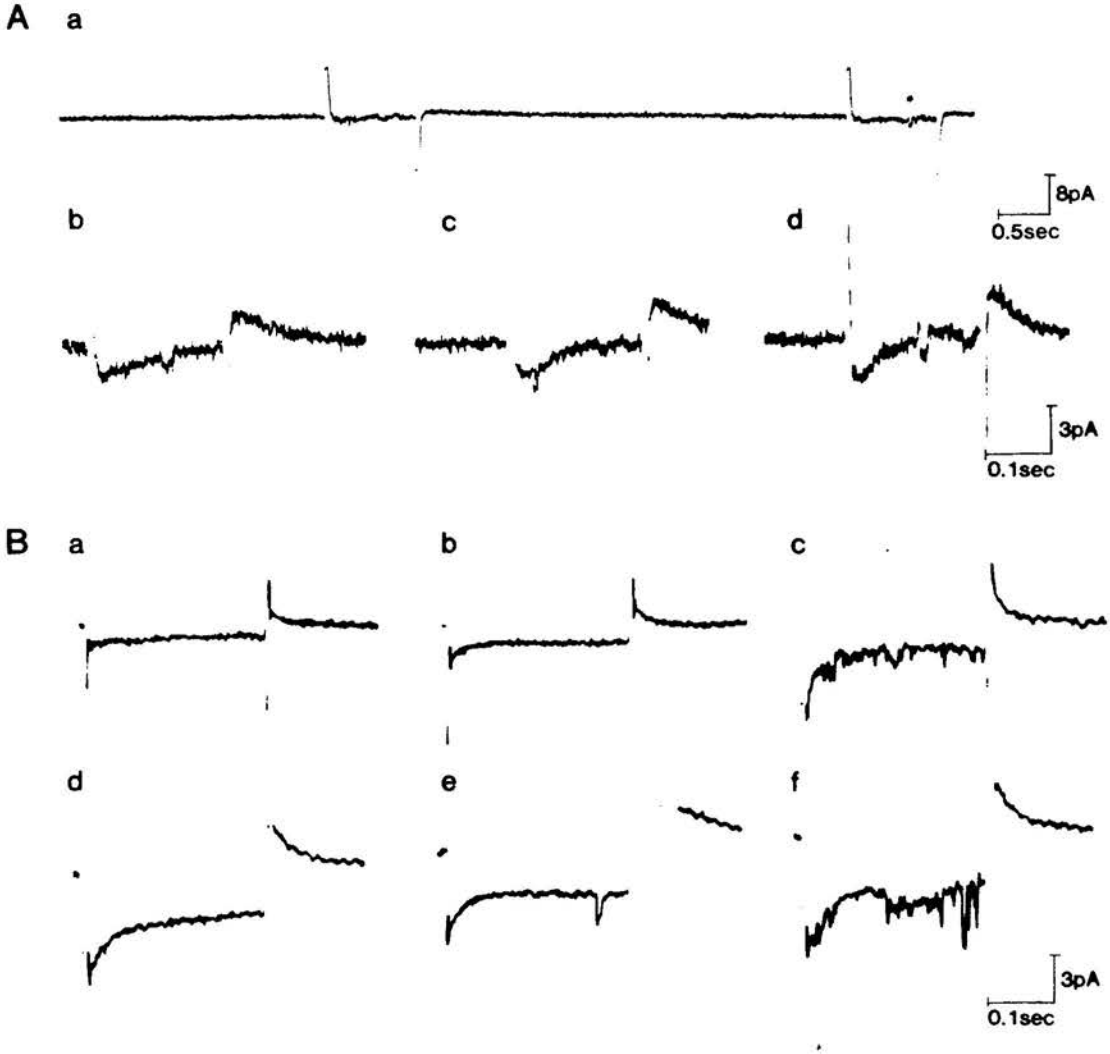
of the egg, (i.e. transpatch potential was 30mV further away from the threshold for activation of these inward currents, than was the rest of the egg membrane). In this situation although a depolarizing pulse elicited an action potential in the rest of the egg, no inward currents or channel openings were observed in the patch. The absence of an opening was not explained by simple probability, (since occasionally "failures" were observed with pipette potentials of -10mV) because repetitive depolarizations, after prolonged intervals did not cause channel opening. When the pipette potential was restored to -10mV, inward currents (marked by an asterisk) were again observed (bottom pair of traces in Figure 4.4B).

4.7 Depolarizing pulses applied across the patch during cell attached patch recording

Once a cell attached patch had been obtained, it was possible to impose depolarizing pulses across the patch itself. Such experiments are illustrated in Figure 4.5. In these experiments, there were no intracellular electrodes. Aa is a pen trace recording of the patch current observed, during two consecutive depolarizing pulses (amplitude = 50mV). During the second of these pulses, an inward current was observed, marked by an asterisk, above the trace. Similarly three other examples are shown as oscilloscope pictures in Ab, Ac and Ad, the depolarizations being 30mV, 44mV and 50mV respectively. There was an increase in inward

Figure 4.5

Patch current traces (Aa is a pen trace and the rest are oscilloscope pictures) recorded during depolarizations of the patch membrane. In A the egg was bathed in Ca4K5, and the patch pipette was filled with Sr60/TEA/CsCl. In B, another egg was bathed in normal solution containing 10mM calcium (Ca10K5) and the patch pipette was filled with TEA-10 CsCl-125 containing 10mM calcium. The composition of this pipette filling solution was mM: TEA,10; CsCl,125; $CaCl_2$,10, buffered with Hepes and KOH to a pH of 7.2 The amplitudes of the depolarizing pulses were 20mV in Ba, 30mV in Ab and Bb, 40mV in Bc, 44mV in Ac and 50mV in the remainder (i.e. Aa, Ad, Bd -Bf). The asterisk in Aa indicates an inward current.


A
a
b
c
d
8pA
0.5sec
3pA
0.1sec
B
a
b
c
d
e
f
3pA
0.1sec

current activity during each of these pulses, when compared to that observed at any time outside the pulse.

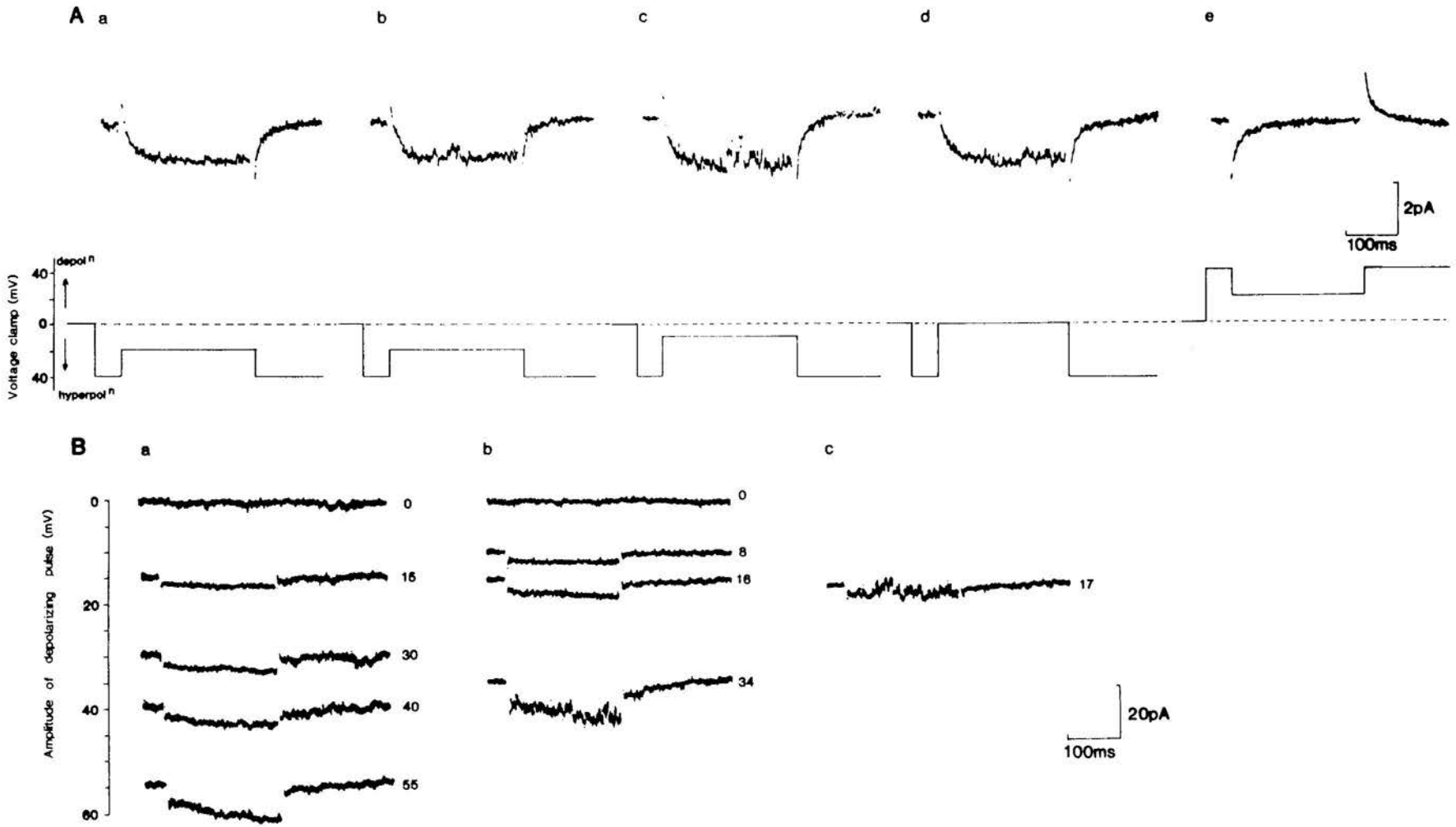
In another similar experiment on another egg, the results of which are shown in Figure 4.5B, many "failures" were observed (i.e. no channel opening during the depolarizing pulses). No inward channel current was observed when the size of the depolarizing pulse was 20mV (Ba) and 30mV (Bb). But inward currents were recorded when the depolarizing pulse was 40mV (Bc) and 50mV (Be and Bf). But occasionally even with a 50mV depolarizing pulse no inward channels were observed (Bd). These experiments would indicate that the threshold for activation of these inward currents was 30-40mV more positive than the transpatch potential. Since the pipette potential was zero, the transpatch potential should be equal to the resting potential of the egg. This is assuming that the physical act of forming the cell attached patch did not alter the potential difference across the patch membrane.

If it is assumed that the threshold for the activation of these inward currents, is equal to the threshold of the electrically evoked action potential, then experiments such as those described above, give an indication of the relative potentials of the threshold and the resting potential in any given egg.

Another similar experiment is shown in Figure

Figure 4.6

A. Oscilloscope pictures of patch current records during various voltage commands across the patch. The voltage commands are drawn schematically below each trace. B. Oscilloscope pictures of patch currents recorded during depolarizations of various amplitudes applied across the patch. The amplitudes of each depolarization are given on the right of each trace. Those recorded in a,b and c were approximately at consecutive half hour intervals. The bathing solution for A and B was Ca10K5 and the patch pipette filling solution was TEA-10 CsCl-125 with 10mM calcium. In this figure all inward currents are indicated as upward deflections (this being the only exception to the usual convention other than the results displayed in Chapter 7).


A
a
b
c
d
e
2pA
100ms
depol n
hyperpol n
Voltage clamp (mV)
40
0
40
B
a
b
c
Amplitude of depolarizing pulse (mV)
0
20
40
60
0
15
30
40
55
0
8
16
34
17
20pA
100ms

4.6A. It should be emphasised at this point that all inward currents are indicated as upward deflections in Figure 4.6 (in the rest of this study except for Chapter 7, inward patch currents are denoted as downward deflections). In this particular egg inward currents were not observed when depolarizing pulses were applied across the patch, so it was assumed that the membrane potential of this egg is more positive than the threshold for the activation of these channels. The following experiments confirmed this. When the patch was hyperpolarized by 40mV (pipette potential = +40mV) and a depolarizing pulse of amplitude greater than 10mV then applied, channels were observed. The amplitude of the depolarizing pulse was 20mV, 30mV and 40mV in Ab, Ac and Ad respectively. An absence of channel opening was observed with a depolarizing pulse of 20mV (Aa). Therefore the resting potential of this egg (or more accurately the transpatch potential) was at least 20mV more positive than the threshold for the activation of these channels.

If the patch was depolarized by 30mV then hyperpolarizing pulses up to 60mV did not initiate channel opening (in Ae the hyperpolarizing pulse was 20mV).

In Figure 4.6B are results of an experiment which can only be described qualitatively. When a cell attached patch was obtained, depolarizing pulses of various magnitudes were applied across it. Patch

current traces of these are shown in Ba, with depolarizing pulses of amplitudes 0, 15, 30, 40 and 55mV. About half an hour later depolarizing pulses of 0,8,16 and 34mV were applied across the same patch and the results are shown in Bb. A further half an hour later a depolarizing pulse of 17mV was applied across the patch, the result of which is shown in Bc. There are two interesting features in this record. Firstly the "noise" at any time outside the pulse is greater in Ba than in Bb or Bc. Secondly, a great deal of channel activity was initiated by a depolarizing pulse of 34mV in Bb. It was not possible to do this even with a depolarizing pulse of 55mV about half an hour earlier in Ba. In Bc a smaller depolarizing pulse (only 17mV) was sufficient to initiate this large increase in channel activity (presumably associated with the initiation of an action potential). A possible interpretation of these results is that the resting potential of a zona-free hamster egg declines (depolarizes) quite rapidly in vitro, hence smaller pulses are needed to elicit an action potential. The assumption being that the threshold remains constant with time. Therefore during the sealing after impalements, although the recorded membrane potential may be increasing (as a result of the egg membrane sealing to the electrode tip) the true resting potential of the egg (i.e. actual potential difference across the egg membrane in the absence of q leak

Table 4.2

Results of whole cell recordings with various bathing solutions, and patch pipette filling solutions. Arrows indicate a decline in the measured membrane potential and the measured input resistance. Some of the patch pipette filling solutions contained 140mM KCl in order to mimic the high intracellular potassium concentration.

Bathing Solution	Patch pipette filling solution (mM)	Measured membrane potential E_m (mV)	Measured input resistance R_m (MΩ)	Seal resistance R_s (MΩ)	Corrected membrane potential E_t (mV)	Corrected input resistance R_t (MΩ)
Ca4K5	KCl=140 EGTA=10 Ca=0 Hepes/KOH	-13	80			
Ca10K5	CsCl=125 TEA=10 Ca=10 Hepes/KOH	-44 -50→-18 -53 -54 -62→	600 1000→250 →100	30,000	-52	1030
	Ca10K5 (90%)	-27 -60	600	1000	-150	1500
	Normal solution Ca=0 EGTA=5	-76→-44	100→50	500	-96	130
	KCl=140 EGTA=10 Ca=0 Hepes/KOH	-73→0 -72→-39→-25	175→ 250→140→25	420 1000	-125 -96	300 330
	KCl=140 EGTA=38 Ca=0 Hepes/KOH	-80→-47→-21	180→70→40	500	-117	230
	KCl=140 EGTA=40 Ca=4 Hepes/KOH	-32→-25→-22 →-18→-15	310→240→190 →130→110			
Sr10K5	Ca10K5 (90%)	-69				

TABLE 4.2

conductance caused by impalement) may actually be declining. This decline being unassociated with the impalement, although the impalements may accelerate the decline. Hence *in vitro* the resting potential of an egg may be changing as a consequence of altering equilibrium potentials of potassium and chloride ions.

The decrease in the baseline noise (i.e. outside the pulse) from Ba to Bc cannot be attributed to an improved seal between the patch pipette and the egg membrane, because the converse was observed (i.e. seal resistance decreased from Ba to Bc).

4.8 Preliminary attempts at measuring whole cell potentials of zona-free hamster eggs (Table 4.2)

In 14 eggs, whole cell potentials were measured of zona-free hamster eggs. These values are tabulated in Table 4.2 and have been called the "measured membrane potentials". In nine of these eggs the input resistance was also noted - called the "measured input resistance". The composition of the bathing and patch pipette filling solutions are all listed in Table 4.2. The mean values of the measured membrane potential and the measured input resistance were -55 ± 20mV, n=14 and 360 ± 310MΩ, n=9 respectively (the ranges were -13mV to -80mV and 84MΩ - 1000MΩ).

These measured values may theoretically be underestimates, because the reciprocal of the measured input resistance is in fact equal to the sum of the reciprocals of the seal resistance and the true input

resistance of the egg. The equivalent circuit being two resistors in parallel (i.e. the seal resistance and the input resistance of the egg) each with its own battery.

$$\text{i.e. } \frac{1}{R_m} = \frac{1}{R_s} + \frac{1}{R_t}$$

where

R_m = measured input resistance

R_s = seal resistance

R_t = true input resistance

Therefore, only when the seal resistance is extremely high compared to the true input resistance of the egg, does the measured input resistance of the egg approximate to the true input resistance of the egg. Hence if the input resistance is underestimated, then so is the measured membrane potential an underestimate of the true membrane potential of the egg. In order to compensate for these underestimates, the measured input resistance and measured membrane potential may be corrected to yield the true input resistance and true membrane potential, as follows. Since

$$E_m = \frac{\frac{E_t}{R_t} + \frac{E_s}{R_s}}{\frac{1}{R_t} + \frac{1}{R_s}}$$

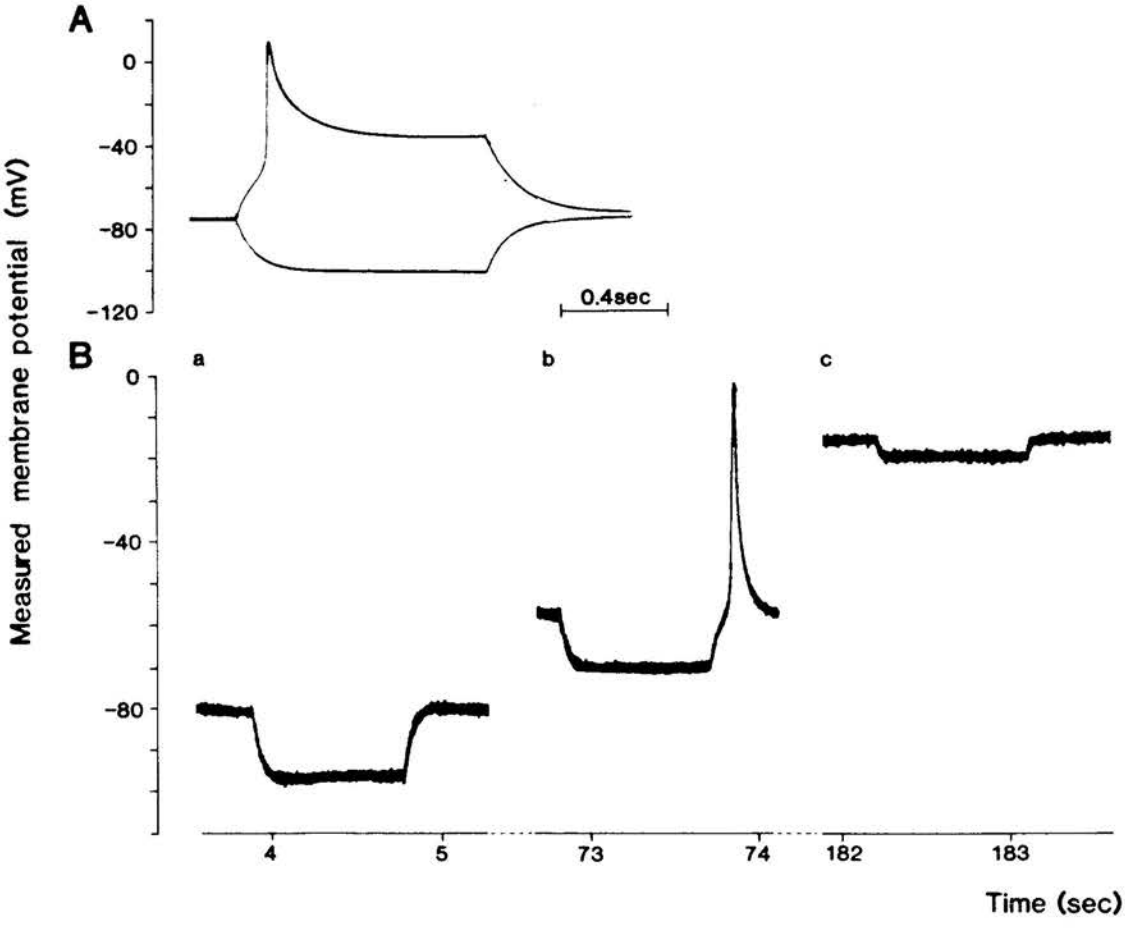
where

E_m = measured membrane potential

E_t = battery of cell (operating across the egg's input resistance), i.e. the true membrane potential of the egg.

Figure 4.7

A. Oscilloscope picture of an electrically evoked action potential during a whole cell recording in an egg bathed in Ca10K5. The patch pipette was filled with normal solution containing 5mM EGTA but no calcium. A hyperpolarizing pulse of the same magnitude as the depolarizing pulse (0.08nA, 0.9 sec) elicited an electrotonic potential (A). B. Oscilloscope pictures of responses evoked during a whole cell recording by passing hyperpolarizing pulses of constant amplitude (0.09nA, 0.9 sec, 0.2Hz) across the egg membrane. The responses a, b (anode break response) and c illustrate the fall in the membrane potential and the input resistance with time (zero time indicates the instant when a whole cell recording was obtained). The measured membrane potentials in a, b and c were -80mV, -58mV and -16mV respectively and the input resistances were 180MΩ, 140MΩ and 40MΩ respectively. This egg was bathed in Ca10K5 and the patch pipette was filled with a solution containing mM: KCl,140; EGTA,38; buffered with HEPES and KOH at a pH of 7.2.


A
0
-40
-80
-120
0.4sec
B
a
b
c
0
-40
-80
4
5
73
74
182
183
Time (sec)
Measured membrane potential (mV)

E_s = battery of seal (operating across seal resistance).

Therefore assuming E_s = 0mV

$$E_m = \frac{E_t R_s}{R_s + R_t}$$

The above "corrections" were applied to the measured input resistance and the measured membrane potential values, in eggs in which measurements of the seal resistance were also obtained. These corrected values are also tabulated in Table 4.2. The assumption made during these calculations is that the seal resistance does not alter, whilst breaking the patch (see 2.3c) - an essential step in the formation of a whole cell recording from a cell attached patch recording. If the seal resistance did change during the procedure, it is likely to have decreased, and hence the underestimates would have been greater.

Assuming that the threshold of the electrically evoked action potentials is -60mV, then the measured membrane potentials indicated that seven of the 14 eggs had high potentials. But the results appear to be inconsistent with the hypothesis that higher membrane potentials were associated with higher input resistances although the results of Fig.4.7B, argue for the idea. For example, one egg bathed in Ca10K5 (patch pipette filled with normal solution, containing 5mM EGTA but no calcium) had a measured membrane potential of -76mV, but a measured input resistance of only 100MΩ

Whereas another egg bathed in the same solution (patch pipette filled with a solution containing mM: CsCl,125; TEA,10; $CaCl_2$,10; buffered with Hepes and KOH) had a measured membrane potential and a measured input resistance of -50mV and 1000MΩ respectively. These values when corrected were -52mV and 1030MΩ. Although in this latter result the patch pipette was filled with a solution containing 10mM calcium and no EGTA (hence cannot be considered an accurate measure of the membrane potential, since the composition of the pipette filling solution is unlike that of the cytosol), the seal resistance was very high (30GΩ). Hence the measured membrane potential approximates to the true membrane potential, i.e. -50mV and -52mV respectively.

Once a whole cell recording had been obtained it was possible to evoke action potentials by passing depolarizing pulses across the egg membrane. One such example of an action potential is shown in Figure 4.7A. A hyperpolarizing pulse of the same magnitude as that used to evoke the action potential elicited an electrotonic response, also shown in Figure 4.7A.

During the above mentioned tabulated experiments, in seven of them the measured input resistance was continually monitored. In all seven a gradual decrease in input resistance was recorded, accompanied by a fall in the measured membrane potential (exemplified in Figure 4.7B) over a period of five minutes. These

decreases are indicated in Table 4.2 by arrows.

Hyperpolarizing pulses of constant amplitude were continuously passed through the patch pipette once a whole cell had been obtained, in order to monitor the input resistance of the egg membrane continuously. Initially an electrotonic potential was observed (Ba), but as the membrane potential and the input resistance of the egg decreased, anode break responses were observed (Bb). A further decrease in the membrane potential and the input resistance of the egg caused the membrane potential to become more positive than the threshold for the anode break response, hence electrotonic potentials were once again observed (Bc).

Since the input resistance decreased as the membrane potential depolarized, a high potential cannot be attributed to a calcium activated potassium conductance (as in Figure 4.1), unless the membrane potential was more negative than the equilibrium potential for potassium. If the membrane potential was more positive than the equilibrium potential for potassium a calcium activated potassium conductance would have caused an increase in the resistance accompanied by a depolarization.

The decrease in the measured input resistance with time is probably due to a decrease in the seal resistance. Therefore while transforming a cell attached patch to a whole cell recording configuration, the seal resistance may not be constant.

4.9 Discussion

In three studies on eggs, various workers have noted that a small proportion of the impalements yielded resting potentials which were susbtantially greater than the average. A similar result has been noted in the present study on zona-free hamster eggs. The three reports are:-

a) the study by Jaffe, Gould-Somero and Holland (1979), in which the authors discarded all eggs (of marine worms) which had resting potentials less negative than -25mV, because they were considered to be "damaged". Of the remainder which had steady resting potentials more negative than -25mV, the mean was 33±6mV (n=133). Out of these 133 eggs, they observed that two had resting potentials more negative than -50mV.

b) the mean resting potential of eggs of Rana pipiens were observed by Cross and Elinson (1980) to be -36±2mV (n=32). But in 8 of these eggs, the membrane potentials were reported to lie in the range -50 to -67mV. However these workers noted no significant difference in the potential measured by one electrode and the potential measured when the egg was impaled with two electrodes. They also reported (although no data was shown) that the current voltage relationship was not linear. Both these observations indicated that the leakage at the electrode tip was small (Hagiwara & Jaffe, 1979).

c) in the study by Miyazaki and Igusa (1981), the resting potential of unfertilized hamster eggs was reported to be -29±7mV (n=120), but 7% of the values lay between -40 and -50mV. The largest observed value was -50mV (input resistance = 400MΩ, temperature = 30 to 32°C). These workers also noted that higher resting potentials were associated with higher input resistances.

The results of these four studies (including the present one) may therefore be an indication that most eggs have undergone an "in vitro deterioration". This may be accelerated by or is caused by the impalement of the first electrode. This deterioration does not appear to affect the fertilizability of these three species of eggs. In such fertilized eggs abnormalities may be seen at much later stages of development.

There are studies which have shown that the resting potentials of eggs, may actually be divided into two groups:-

a) two groups of resting potentials have been observed in ascidian oocytes. One group has a range -20 to -35 mV and the other -80 to -90mV (Dale, de Santis & Ortolani, 1983; 1.18). These authors reported that both classes of oocytes cleaved equally well giving rise to apparently normal blastulae. But they suggest that the development through early larval stages may indicate some differences between the two populations.

b) similarly the resting potentials of the tunicate, Clavelina could assume either of two stable values,

approximately -70 and -30mV (Thompson & Knier, 1983; 1.18).

c) two groups of resting potentials have been reported for sea urchin eggs. One group having resting potentials of -10mV (Steinhardt et al., 1971) and another group about -70mV (Jaffe & Robinson, 1978). Measurements of unidirectional fluxes have indicated that the resting potentials are about -70mV (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979; 1.13). In the sea urchin and starfish at least, the two groups of membrane potentials have been attributed to properties of the potassium permeability of the eggs (Hagiwara & Jaffe, 1979). There was no indication in the present study that the resting potential fell into two groups.

Experiments on amphibian eggs have indicated that the resting potentials are higher if the eggs are isolated from the follicles, compared to those obtained followng injections (Ziegler & Morrill, 1977; Wallace & Steinhardt, 1977). All experiments performed in this study were on eggs obtained by superovulation, induced by hormonal injections. It would therefore be interesting to record the resting potentials of some "naturally ovulated" eggs.

Inward currents were observed at sufficiently hyperpolarized values of the transpatch potential (at least about -70mV: Fig.4.3). Due to the composition of the solution in the pipette it was concluded that these currents were either chloride or strontium (via calcium

channels), since no sodium or potassium was present in the pipette filling solutions. Furthermore, the pipette filling solution contained TEA and caesium chloride. Outward currents were never observed, hence it was concluded that these inward currents were due to the influx (into the egg, from the pipette filling solution) of strontium via calcium channels. Such inward currents were observed in synchrony with anode break responses (Fig.4.3) and electrically evoked action potentials (Fig.4.4). Evidence for the existence of calcium channels in mouse eggs was initially presented by Okamoto et al. (1977). These workers noted a calcium inward current during voltage clamp experiments, correlated with anode break responses. Okamoto et al (1977) suggested that in the mouse egg the measured resting potential is a depolarized value of the true resting potential, caused by a leakage due to the penetration of the electrode. They indicated that at this depolarized level, the calcium channels are inactivated.

The imposition of pulses across the patch (either by means of an intracellular electrode, or by a transient shift of the pipette potential) appeared to be a better method for observing calcium channels in this preparation. In similar experiments on chromaffin cells (Fenwick et al., 1982b) it was possible to obtain estimates of the actual holding potential during cell attached patch recording, by comparing data obtained in isolated patches with that

obtained in cell attached patches. In the present study this was not possible because no recordings were made with isolated patches.

The maximum size of the inward current observed by Okamoto et al. (1977) in sodium free media containing 20mM strontium, at 33°C was $9.70 \pm 1.97 \times 10^{-9}$A. Assuming that the amplitude of a single channel calcium current is 1×10^{-12}A (see 3.6) and the diameter of the egg is 80μm, then the number of calcium channels per μm^2 membrane surface area equals

$$\frac{9.7 \times 10^{-9}}{1 \times 10^{-12}} \times \frac{1}{4 \times \pi \times (40 \times 10^{-6})^2}$$

$$\simeq 0.5 \mu m^{-2}$$

where $4 \times \pi \times (40 \times 10^{-6})^2$ is the surface area of the egg, treating it as a sphere of diameter 80×10^{-6}m. This value for the channel density may be overestimated, because of an underestimate in the surface area of the egg, due to the presence of the microvilli. Assuming an egg capacitance of 660pF (Georgiou et al., 1984), and a specific membrane capacitance of $1 \mu F cm^{-2}$, then the actual surface area of the egg is calculated to be 6.6×10^{-4} cm^{-2}. Whereas the calculation of the membrane area assuming the egg to be a sphere of diameter 80×10^{-6}m, gives a value of 2×10^{-4} cm^2 (approx). Hence the actual patch area is apparently underestimated by about three fold. Therefore the calcium channel density is about $0.2 \mu m^{-2}$,

taking into account this underestimation. The channel density compares well with that obtained in rat cardiac cells - 0.1 to 0.5 channels per μm^2 (Reuter et al., 1982). Assuming a patch pipette tip internal diameter of 2 μm, then the surface area of the egg membrane enclosed by the pipette is about 3-4 μm^2. Therefore theoretically no more than one calcium channel should be observed in each patch (in some patches no channels may be observed). Although it is likely that the patch area is larger than that mentioned above, due to the suction applied to the interior of the pipette (to facilitate giga seal formation, see 2.3b). The sizes of the fluctuations observed (assumed to be the summation of discrete current events), during depolarizing pulses passed across cell attached patches, appeared to agree with this (Fig.4.5).

The major problem encountered during whole cell recording experiments was the small seal resistances obtained between the patch pipette and the egg membrane. In six experiments out of seven they were less than 1000 MΩ [compared to 10-50GΩ in Fenwick et al (1982a); 10-100GΩ in Hagiwara and Ohmori (1982)]. Therefore the experiments described in the present study cannot strictly be called "tight seal whole cell recording" (Marty & Neher, 1983).

Other possible causes of error during the whole cell recording experiments may have been:-

a) incompatability of the pipette filling solution with

the egg's intracellular environment, particularly with regards to the calcium buffering capacity.

b) a gradual exchange of ions between the pipette and the cell interior, may cause a drift in the junction potential.

c) a loss of cytoplasmic factors out of the cell, may affect the properties of the cell membrane. As mentioned in Chapter 3, calcium currents have been shown to decline irreversibly, after about 15 minutes of whole cell recording of chromaffin cells (Fenwick et al., 1982b).

The decrease in the membrane potential and the input resistance noted in the present study after the formation of a whole cell recording may be related to the "rundown" effect observed by Fenwick et al. (1982b; described in 3.8).

CHAPTER 5 INTRACELLULAR RECORDINGS DURING FERTILIZATION OF ZONA-FREE HAMSTER EGGS BATHED IN NORMAL AND HIGH POTASSIUM SOLUTIONS CONTAINING VARYING AMOUNTS OF CALCIUM

5.1 Methods

Section A Fertilizations in normal potassium solutions

5.2 Homologous fertlizations of low potential hamster eggs

5.3 Homologous fertilizations of low potential hamster eggs, current clamped at high potentials prior to insemination

5.4 Homologous fertilizations of two high membrane potential hamster eggs

5.5 Heterologous fertilizations of zona-free hamster eggs with mouse sperm

Section B Fertilizations in high potassium solutions

5.6 Homologous fertilizations of low potential hamster eggs current clamped at high potentials prior to insemination

5.7 Homologous fertilizations of low potential hamster eggs current clamped at high potentials prior to insemination in low sodium solutions

Section C Analysis of results

5.8 Membrane potentials and input resistances of zona -free hamster eggs

5.9 Numbers of successful fertilizations performed in the various solutions

5.10 Different types of electrical responses observed during fertilization of zona-free hamster eggs bathed in normal and high potassium solutions

5.11 Frequency of trhs or trds observed at different membrane potentials

5.12 Durations of various types of responses observed during fertilization of zona-free hamster eggs with hamster sperm

5.13 Potentials at the peaks of the slow responses obtained during fertilization and their reversal potentials

5.14 Spike peaks of trd spikes, electrically evoked action potentials and sperm evoked action potentials

5.15 Spike peaks of consecutive trd spikes

5.16 Peak potentials and amplitudes of fsds

5.17 Reversal potentials of fsds

5.18 Predepolarizations prior to trds, trd spikes, fsds and sperm evoked action potentials accompanying sperm fusions

5.19 Is there any depolarizing event prior to the first trh recorded in an egg fertilized at low

membrane potentials?

5.20 Rate of change of potential of the slow and fast responses observed during fertilization

5.21 Responses occurring immediately before or immediately after trhs or trds, which were not associated (closely in time) with sperm fusions

a) trhs followed by after depolarizations

b) trds preceded by hyperpolarizations

c) trds with after hyperpolarizations

5.22 A comparison of the thresholds of the sperm and electrically evoked action potentials

5.23 Plateau potential levels of sperm evoked action potentials

5.24 Brief action potentials

5.25 Spontaneous action potentials

5.26 Discussion

In this chapter are described fertilization experiments performed using intracellular electrodes, in eggs bathed in:-

i) normal solution Ca2K5

ii) high potassium solution Ca2K25

iii) either (i) or (ii) with elevated calcium. In these solutions the concentration of calcium was greater than 2mM. Equiosmolarity was maintained (the same as normal) by reducing sodium chloride by the same amount. Otherwise the composition of these solutions was the same as either normal or high potassium solutions.

iv) a few results are also presented of eggs bathed in high potassium solution containing no sodium chloride. In these solutions the sodium chloride has been replaced by either choline chloride or lithium chloride (see 2.4c). Such solutions have been called Ca2K25(choline) and Ca2K25(lithium) respectively.

The results have been discussed in three sections. In Section A are results obtained in solutions containing normal potassium (ie. concentration of potassium is 5mM). These include results in normal solution and those in normal solution with elevated calcium's (ie. Ca4K5, Ca12K5, Ca42K5, and Ca57K5). Also described in this section are a few results of heterologous fertilizations of hamster eggs with mice sperm (bathed in either Ca2K5 or Ca4K5).

In Section B are the results obtained from eggs bathed in high potassium solutions (ie. concentration

of potassium is 25mM). These include results from eggs bathed in the "standard" high potassium solution (ie. Ca2K25) and from eggs bathed in high potassium solutions with elevated calcium's (ie. Ca4K25, Ca7K25 and Ca10K25). One result was also obtained from an egg bathed in Ca7K40, in which the concentration of calcium chloride, sodium chloride and potassium chloride was 7, 80 and 40 mM respectively. Also described in this section are the results obtained in low sodium solutions ie. Ca2K25(choline) and Ca2K25(lithium).

Finally in Section C, the results from Section A and Section B are analysed.

5.1 Methods

A zona - free hamster egg was impaled and subsequently inseminated as described in Chapter 2. All experiments described in this chapter were performed on zona free hamster eggs. The majority of experiments involved fertilizations with hamster sperm although in a few experiments shown, mice sperm were used.

SECTION A

FERTILIZATIONS IN NORMAL POTASSIUM SOLUTIONS

5.2 Homologous fertilizations of low potential hamster eggs

As described in 4.1, low potential eggs have been defined in this study as being eggs whose membrane potential is more depolarized than the threshold for

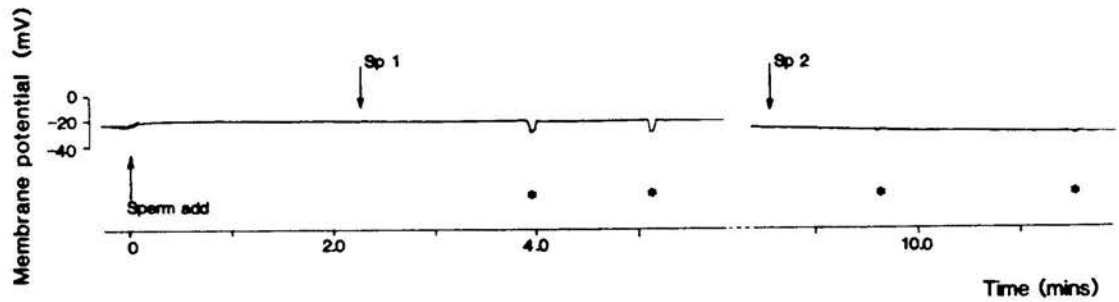
the electrically evoked action potential (high potential eggs being defined as those in which the membrane potential is more hyperpolarized than the threshold for the electrical action potential).

It has been reported that the hamster eggs with low membrane potentials undergo transient recurring hyperpolarizations during fertilization (Miyazaki & Igusa, 1982; Igusa et al., 1983).

Such responses in low potential eggs were observed in this study also. One such example is shown in Figure 5.1. The membrane potential of this egg was -18mV and it s input resistance was 30MΩ (the bathing solution was normal ie. Ca2K5). At zero time sperm were added to the chamber (throughout the whole of this study, zero time is denoted as being the instant of sperm addition). After 2.2 minutes one of the attached sperm became straight and immotile (ie. a sperm had fused with the egg (see 2.5)) as indicated by the first arrow above the pen trace in Fig 5.1. This was accompanied by a small transient depolarization observed on the pen trace. A second sperm fusion was observed at 8.6 minutes. In all, six transient recurring hyperpolarizations were observed (for the remainder of this study transient recurring hyperpolarizations have been abbreviated to trh's). It was found in this recording that trh's did not correlate with the instant of sperm fusion (at least as indicated by sperm straightening). The first trh occurred 1.7 minutes

Figure 5.1

A pen trace record showing the intracellular recording from a zona-free hamster egg, during fertilization with hamster sperm. The egg was bathed in normal solution. Sperm were added at zero time, as indicated by the arrow below the pen trace. Two sperm were observed to fuse with the egg at times 2.2 and 8.6 minutes after sperm addition (as shown by the arrows above the pen trace). Six transient recurring hyperpolarizations (indicated by asterisks) were observed at 3.9, 5.1, 6.8, 8.3, 9.6 and 11.5 minutes (the third and fourth of these is not shown). The egg was confirmed to be dispermic by subsequent histology.


Membrane potential (mV)
0
-20
-40
Sp 1
Sp 2
Sperm add
0
2.0
4.0
10.0
Time (mins)

after the first sperm fusion. Similarly the first trh to occur after the second sperm fusion (this was actually the fifth trh recorded in this egg) was 1.0 minutes after the second fusion.

As observed by the workers mentioned above, sperm fusion did not elicit an obvious electrical response, at the instant of fusion in such low potential eggs. Although a very small transient depolarization (2-3 mV approximately in amplitude) appeared to accompany the first sperm fusion, no such response was seen at the instant of the second sperm fusion.

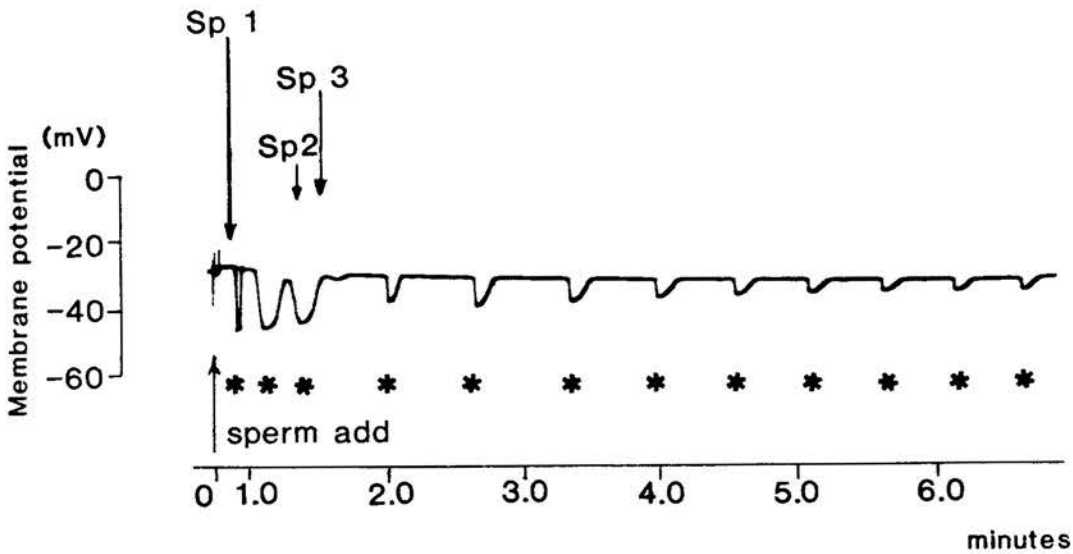
A gradual hyperpolarization was noted during the length of recording shown in Fig.5.1. Initially, before the first sperm fusion , the membrane potential of the egg was -18 mV, whereas at the end of the recording (after the sixth trh) the membrane potential was -26 mV. Such a hyperpolarizing shift (h.s.) is in agreement with the results of previous workers (Igusa & Miyazaki, 1983).

The duration and amplitude of successive trh's decreased, for example, the duration and amplitude of the first and sixth trh's (shown in Fig.5.1) were 7 seconds, 9 mV and 3 seconds, 2 mV respectively. The trh's in Fig.5.1 appear to have secondary depolarizing phases, which have been called "after depolarizations" and are discussed later in this chapter.

Another example of a fertilization of a low potential egg is shown in Fig.5.2.This egg was bathed in Ca42K5 (the concentration of $CaCl_2$, NaCl and KCl in

Figure 5.2

Pen trace of an intracellular recording during fertilization of an egg bathed in Ca42K5. Three sperm fused with the egg at the points marked by arrows above the pen trace. A total of 27 trhs were recorded in this egg, the first 12 of which are displayed (marked by asterisks). The time of sperm addition is also shown. Note the slower chart speed for the first minute of the record shown. The membrane potential and input resistance of this egg prior to insemination was -30mV and 190MΩ. Histology confirmed a trispermic fertilization of the zona-free hamster egg with hamster sperm.


Sp 1
Sp 3
Sp2
(mV)
0
-20
-40
-60
Membrane potential
sperm add
0
1.0
2.0
3.0
4.0
5.0
6.0
minutes

this solution was 42, 60 and 5mM respectively, otherwise the composition of this solution was the same as the normal solution). This egg was fertilized by three sperm, the times of the suspected fusions being 0.5, 1.3 and 1.5 minutes after sperm addition. From this record it is not possible to discriminate the electrical event correlated with each fusion, because the three fusions occurred so soon after sperm addition (the first fusion was only 30 seconds after adding sperm), and also because they occurred so close to each other.

In this recording 27 trh's were observed, only twelve of which are shown (marked by asterisks in Fig.5.2). A hyperpolarizing shift was observed (the membrane potential before the first trh was -30mV whereas that after the twelvth trh was -35mV). The duration of each successive trh decreased (duration of the first trh was 13 sec., that of the eleventh trh was 7 sec.), as did the amplitude (amplitude of the first trh was 19mV, whereas that of the eleventh trh was 3mV). These trh's have been shown by previous workers to be due to a calcium activated potassium conductance (Igusa & Miyazaki, 1983) with a reversal potential of about -80mV. They also showed that if an egg was current clamped to a potential more hyperpolarized than the reversal potential, then transient recurring depolarizations were observed, also due to a calcium activated potassium conductance.

As indicated by Trautwein & Dudel (1958) values for the conductance, g, and the reversal potential, e, at the peak of a response may be derived from a single record of the kinds shown in Fig.5.20. Relations equivalent to those of Trautwein & Dudel are derived as follows. Evidently for a current pulse I, giving electrotonic potentials P and p at rest and during the response, respectively, we have

$$P = \frac{I}{G} \quad \text{and} \quad p = \frac{I}{G+g}$$

where G is the resting conductance. For a resting potential E and a response v, we find

$$g = \left(\frac{P-p}{p}\right) G$$

and

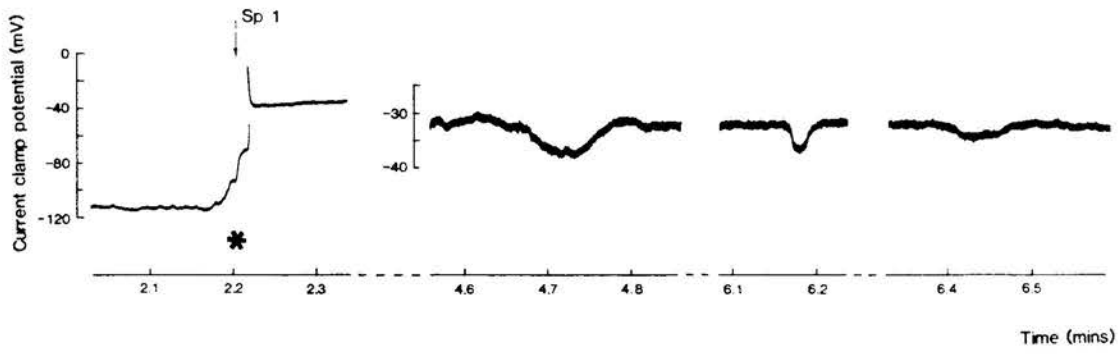
$$e = \left(\frac{P}{P-p}\right) v+E$$

The relation between current and voltage is linear in the range 0 to -150mV, as found originally by Miyazaki & Igusa (1982) for hamster eggs. Hence the above relationships are valid since resting conductance is independent of current and membrane potential. The reversal potentials of trhs calculated during this study are discussed in 5.13. They were found to be in close agreement with those of previous workers (Miyazaki & Igusa, 1982).

One might therefore expect the amplitudes of successive trhs to decrease as the membrane potential hyperpolarizes and hence approaches the reversal potential for these responses. The peak potential of each successive response actually became more

Figure 5.3

Oscilloscope pictures of electrical events recorded during fertilization of a zona-free hamster egg with hamster sperm. The egg prior to insemination was current clamped at a potential of -114mV. The egg bathed in Ca4K5 was fertilized by one sperm. The sperm fusion was synchronous with an action potential which had a plateau (the plateau was maintained, i.e. did not return to the initial current clamp potential before the end of the experiment). There were three trhs superimposed on this plateau, the oscilloscope pictures of which are also shown (the gain of which are greater than the gain of the oscilloscope picture showing the action potential). Preceding the action potential there were two small depolarizations which have been called predepolarizations and are marked by an asterisk.


Sp 1
Current clamp potential (mV)
0
-40
-80
-120
-30
-40
2.1
2.2
2.3
4.6
4.7
4.8
6.1
6.2
6.4
6.5
Time (mins)

depolarized. The peak of the first trh was -46mV, whereas that of the eleventh was -38mV.

5.3 Homologous fertilizations of low potential hamster eggs, current clamped at high potentials prior to insemination

Unlike the experiments described in 5.2 above, the following experiments were performed on eggs current clamped to high potentials (i.e. current clamped to potentials below the threshold for the electrically evoked action potential), before insemination.

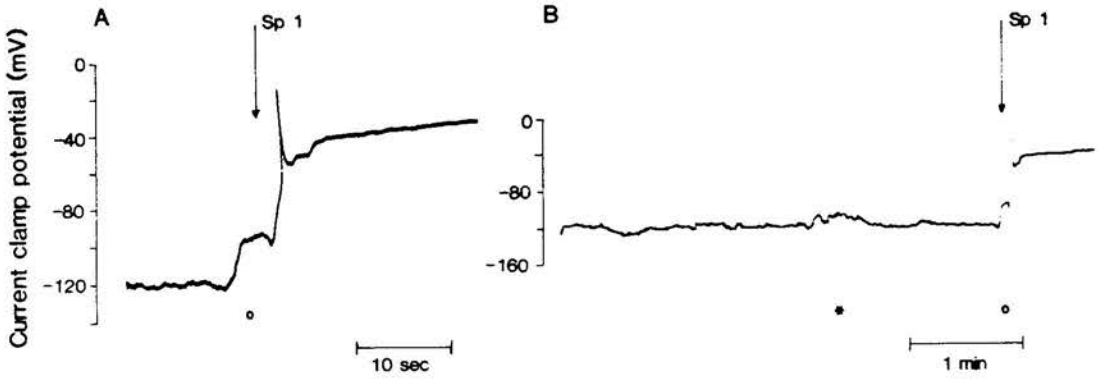
One such example is shown in Fig.5.3. In this a zona - free hamster egg (bathed in Ca4K5) was current clamped to a potential of -114mV, before insemination. A sperm was then observed to become straight and immotile approximately 2.2 minutes after adding the sperm to the chamber. No further fusions were observed in this egg, and subsequent histology showed it to be monospermic. This sperm fusion was accompanied by an action potential shown in Fig.5.3. This action potential with a spike peak of -10mV plateaued at -38mV, and superimposed on this plateau were three trh's (also shown in Fig.5.3) at times 4.7, 6.2 and 6.4 minutes after sperm addition.

Such action potentials have been called sperm evoked action potentials to distinguish them from the electrically evoked action potentials.

Preceeding the action potential shown in Fig.5.3, there appeared to be two distinct depolarizing phases

Figure 5.4

A. Oscilloscope picture of a sperm evoked action potential, recorded in a zona-free hamster egg fertilized by hamster sperm. The egg was current clamped at -120mV and bathed in Ca4K5. B. Pen trace recording of the current clamp potential prior to the sperm evoked action potential shown in A. The start of the depolarization (marked by an asterisk) observed at 1.7 minutes before the action potential corresponded to sperm attachment to the egg. The sperm became straight and immotile at the point marked by the arrow. The action potential was preceded by a predepolarization (marked by an open circle). No other electrical events were observed in this egg, which was shown by histology to be fertilized by only one sperm.


A
Sp 1
0
-40
-80
-120
Current clamp potential (mV)
10 sec
B
Sp 1
0
-80
-160
1 min

which have been called predepolarization events (marked by an asterisk below the oscilloscope picture), and are discussed in Section C of this chapter.

The threshold of this sperm evoked action potential was -70mV, which is similar to the threshold of electrically evoked action potentials (see 5.22). The amplitudes and durations of the three trh's were in order 6mV, 10 seconds; 4mV, 2 seconds and 2mV, 5 seconds.

In another monospermic egg bathed in the same solution (Ca4K5), sperm fusion was again accompanied by an action potential, but although it had a maintained plateau, the action potential was not followed by any trh's. The egg had been current clamped at -120mV, the threshold, peak and the plateau of the sperm evoked potential were -66, -18 and about -46mV respectively. This sperm evoked action potential also showed a predepolarization (Fig 5.4, marked by an open circle). Fig 5.4B shows the pen trace recording obtained in this experiment. It displays 3.8 minutes of recording prior to the sperm evoked action potential. About 1.7 minutes before the action potential, there was a small depolarization (marked by an asterisk, about 12mV in amplitude, lasting about 30 seconds) the start of which corresponded to attachment of the sperm to the egg. But the sperm did not actually become straight and immotile until the predepolarization observed just before the action potential (marked by an open circle in Fig 5.4A

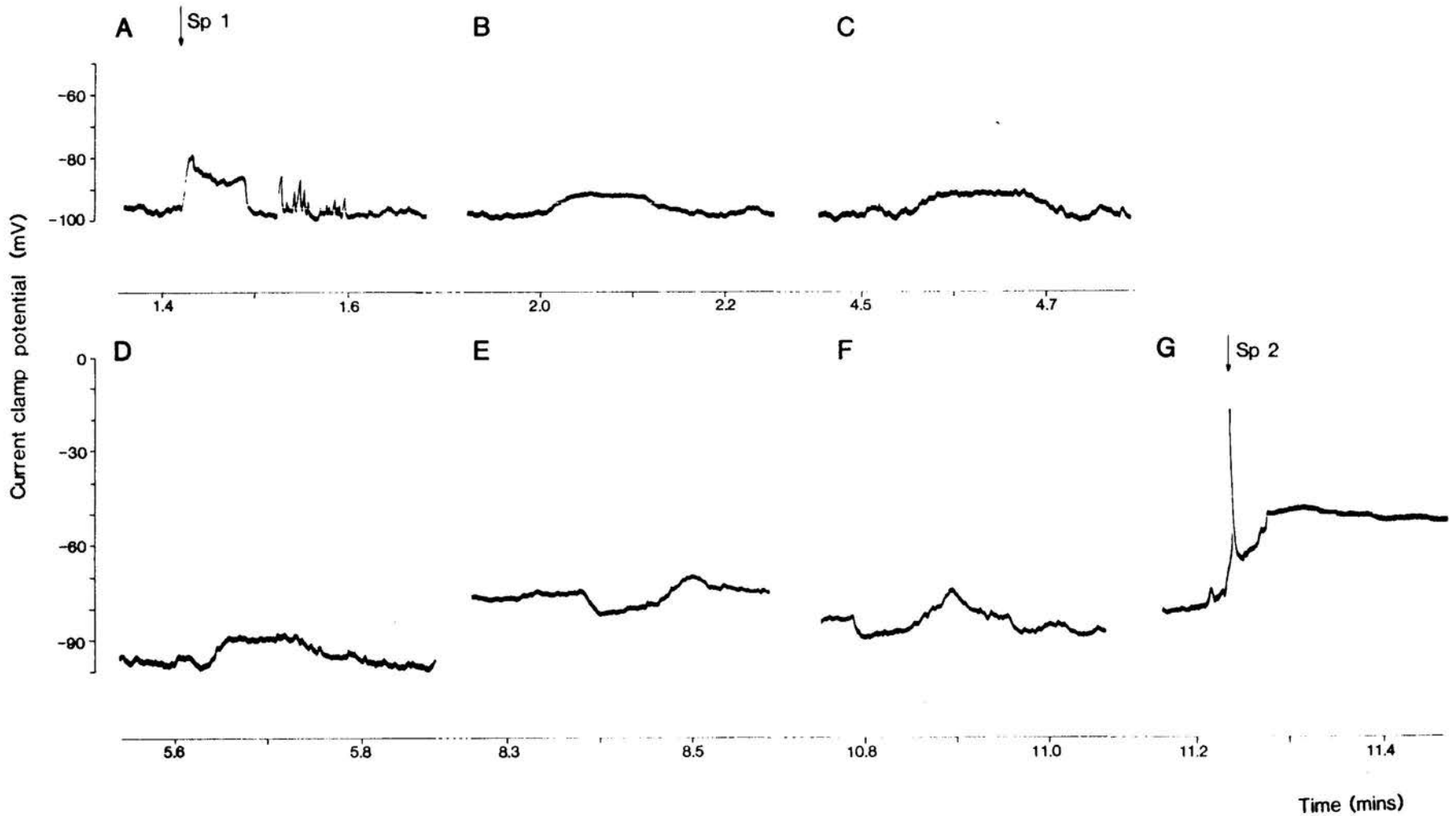
and 5.4B). The plateau potential level of -46mV, indicated above for this egg, was the initial value, almost immediately after the repolarization of the spike. Subsequently the egg depolarized.

Figures 5.3 and 5.4 are two examples in which sperm fusion was seen electrically in the form of sperm evoked action potentials. But often sperm fusion in eggs current clamped at high potentials was seen as a fast subthreshold depolarization (abbreviated to fsd) without a following action potential. It has been called a "fast" response because its rate of change of potential is greater than that of a trh, the latter being called a "slow" response. Rates of change of potential of these sperm evoked responses are discussed and analysed in Section C of this chapter. An fsd has been called subthreshold, because its peak potential is more hyperpolarized than the threshold for the sperm evoked action potential.

An example of an fsd is illustrated in Fig 5.5A. This egg had a low membrane potential, it was bathed in Ca4K5, current clamped to a potential of -93mV and then inseminated. The fusion of the first sperm in this egg was observed electrically as an fsd (shown in Fig 5.5A). This occurred 1.4 minutes after insemination. It had an amplitude of 17mV, with a peak potential of -79mV, and lasted for 4 seconds. This was followed by some very brief but fast excursions of the current clamp potential (Fig 5.5A) which have been assumed to be correlated with fusion, since they are not normally

Figure 5.5

Oscilloscope pictures showing electrical events recorded during homologous fertilization of a zona-free hamster egg bathed in Ca4K5. This egg was fertilized by two sperm. The first sperm fusion at 1.4 minutes was accompanied by an fsd shown in A, whereas a second at 11.2 minutes elicited an action potential, shown in G. The first fusion was recorded whilst the egg was current clamped at -93mV (A). This was then followed by three trds shown in B,C, and D. The current clamp was then reduced, such that the current clamp potential was -70mV. At approximately this potential two trhs were recorded (shown in E and F), followed by a sperm evoked action potential, which accompanied the second fusion (G). The two trhs shown in E and F had prominent after-depolarizations.


A
Sp 1
B
C
-60
-80
-100
1.4
1.6
2.0
2.2
4.5
4.7
D
E
F
G
Sp 2
0
-30
-60
-90
5.6
5.8
8.3
8.5
10.8
11.0
11.2
11.4
Current clamp potential (mV)
Time (mins)

observed in unfertilized eggs.

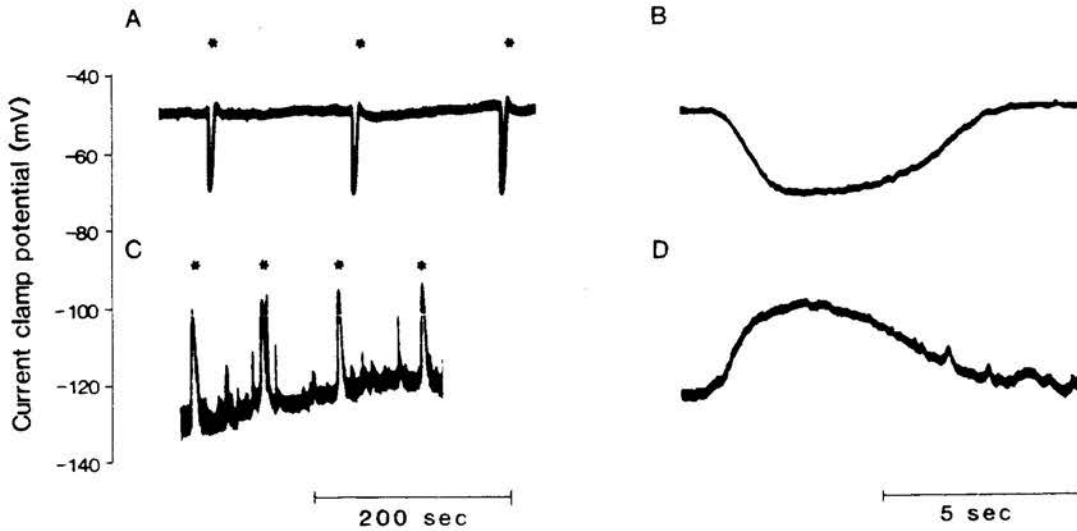
At 2.0 minutes a small transient depolarization was observed (Fig 5.5B). This is a transient recurring depolarization, the rate of change of potential of which visibly resembles that of trh's. Hence this sort of response has also been classified as a slow response, like the trhs (to distinguish it from the fsd's which are "fast" responses). Two more transient recurring depolarizations were observed at 4.6 and 5.6 minutes, shown in Fig 5.5C and Fig 5.5D respectively.

After the trd shown in Fig 5.5D, the constant hyperpolarizing current passed through the electrode was reduced, so that the egg was current clamped at -70mV (previously it had been current clamped at -93mV). Then at 8.4 and 10.8 minutes, two trh's were observed (shown in Fig 5.5E and Fig 5.5F respectively). But interestingly each of these trh's had prominent after depolarizations.

Then at about 11.2 minutes with the egg still current clamped a second sperm fusion was suspected. At approximately this time an action potential was observed, which is believed to have been evoked by this second fusion. This sperm evoked action potential plateaued at about -50mV, it's threshold and peak were -64mV and -15mV respectively (shown in Fig 5.5G). In this no prominent predepolarization event occurred. This may be because the current clamp potential was so close to the threshold for the sperm evoked action

Figure 5.6

A. Three trhs superimposed on the plateau of a sperm evoked action potential (shown in Figure 5.5G). Each trh is marked with an asterisk. The first of these trhs is shown in B. C shows four trds observed in the same egg, whilst current clamped at about -130mV. Each trd is marked with an asterisk. The last of these trds is shown in D. The egg was bathed in Ca4K5.


A
B
C
D
Current clamp potential (mV)
-40
-60
-80
-100
-120
-140
200 sec
5 sec

potential, a predepolarization was not seen electrically.

Prior to the sperm evoked action potentials shown in Figs. 5.3 and 5.4, prominent predepolarizations were noted. The fact that the predepolarizations were large in those examples might have been related to the fact that the current clamp potentials (-114 and -120mV respectively) were a lot more hyperpolarized than the threshold for the sperm evoked action potential (compared to the example illustrated in Fig 5.5, in which the current clamp potential was -80mV just prior to the action potential).

In Fig.5.5G it is noted that the spike repolarizes to below the plateau, as seen in Fig 5.4. But unlike the sperm evoked action potential shown in Fig 5.4, the plateau in Fig 5.5G hyperpolarized gradually.

After the sperm evoked action potential shown in Fig 5.5G, three trhs were recorded, which have been shown in 5.6A (these three trhs were actually superimposed on the plateau of the action potential). The times of the three trhs (all had after depolarizations) were 12.8, 15.3 and 17.8 minutes. Each trh is denoted by an asterisk in Fig 5.6A, the first of these is shown on a faster time base in Fig 5.6B (amplitude, peak potential and duration were 21mV, -71mV and 7 seconds respectively). Shortly after the third trh shown in Fig 5.6A the egg was current clamped at a potential of about -130mV. At this potential four trd's were noted, shown in Fig 5.6C, each one of which

is marked with an asterisk. The last of these trds shown in Fig 5.6C is shown in Fig 5.6D, on a faster time base (amplitude, peak potential and duration were 25mV, -105mV and 7seconds respectively).

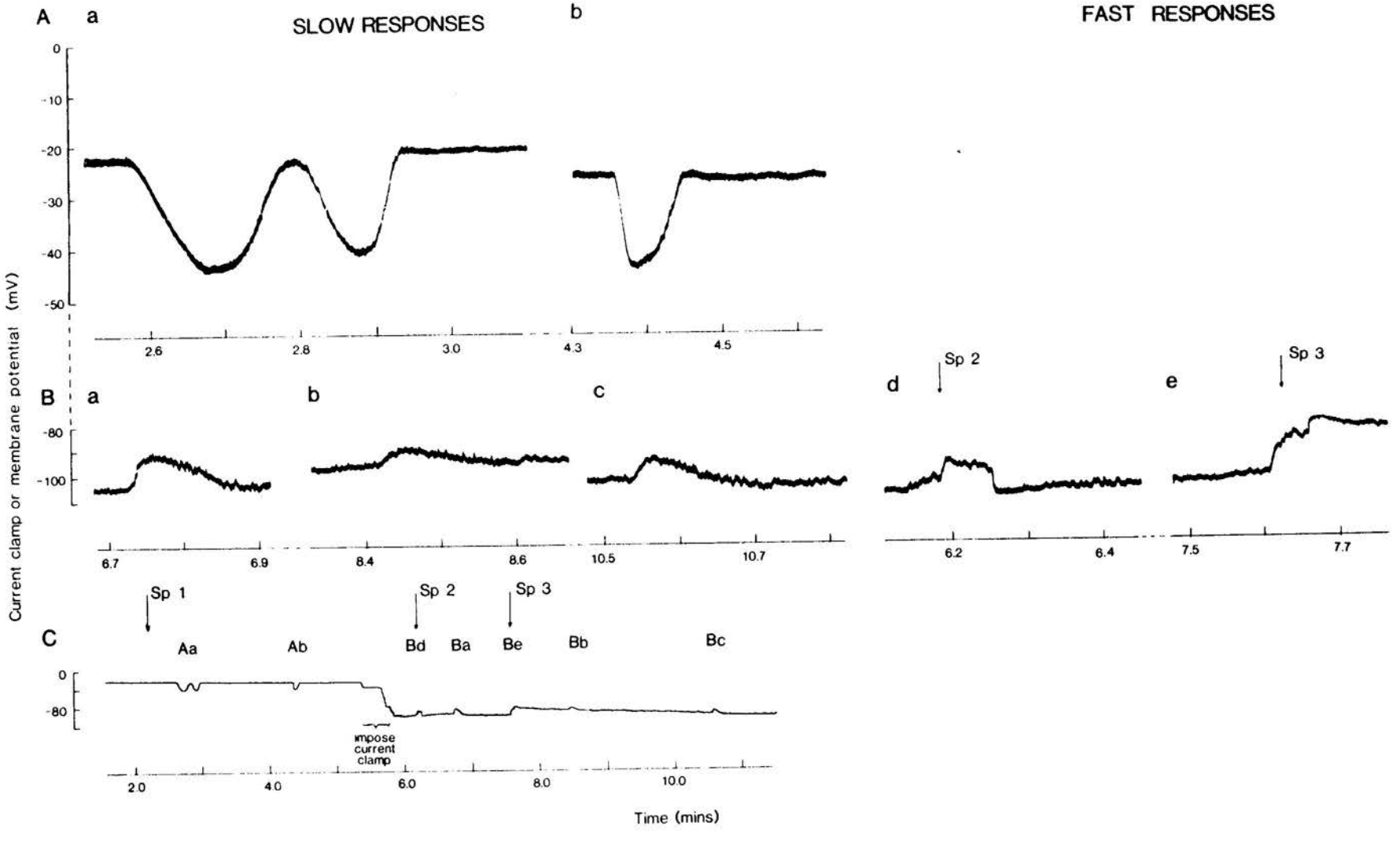
These results indicate that trhs and trds occur by the same ionic mechanism and that they reverse at some potential between -71mV (peak potentials of trh shown in Fig 5.6B) and -105mV (peak potential of trd shown in Fig 5.6D). Previous workers have shown that trhs are due to a calcium activated potassium conductance (Igusa & Miyazaki, 1983; Georgiou et al., 1983).

The baseline shown in Fig 5.6C gradually depolarizes. This is because a large amount of current was being injected into the egg, in order to current clamp it at such a hyperpolarized potential (-130mV). The egg was unable to tolerate this large current, and presumably because of a decrease in the seal resistance, or even damage to the egg membrane caused by this current, the egg gradually depolarized.

The intervals between the three trhs shown in Fig 5.6A were 147 seconds and 148 seconds. Similarly the intervals between the four trds shown in Fig 5.6C were 73, 80 and 85 seconds. These results therefore indicate that the interval between successive slow responses (ie. trhs or trds) decreases at more hyperpolarized potentials. This has been noted by other workers also (Igusa & Miyazaki, 1983).

Figure 5.7

Homologous fertilization of a zona-free hamster egg bathed in Ca57K5. The egg was inseminated at it's membrane potential which was -22mV. Then at 5.3 minutes the egg was current clamped to a potential of -106mV. Each electrical event shown in the pen trace C is shown in the form of an oscilloscope picture in either A or B. Letters above the responses shown in C indicate the labelling of the corresponding oscilloscope pictures in A or B. In A are shown the electrical events observed while the egg was at its membrane potential (low) i.e. prior to current clamp. In B are shown the electrical events observed whilst the egg was current clamped at a high potential (-106mV). The egg was fertilized by three sperm (confirmed by histology). The first fusion occurred at a low membrane potential, in which case no electrical event was noted. The other two fusions occurred whilst the egg was current clamped at a high potential in which case fsds were observed (Bd and Be respectively). Aa and Ab show the trhs recorded at the low membrane potential, Ba - Bc show the three trds observed while the egg was current clamped at a high potential. The responses have been classified into two groups, firstly slow responses (i.e. trhs and trds) and secondly fast responses (i.e. fsds). No fast responses were observed at low potential. The instants of the three sperm fusions are indicated by arrows in C, Bd and Be.


A
a
SLOW RESPONSES
b
FAST RESPONSES
0
-10
-20
-30
-40
-50
2.6
2.8
3.0
4.3
4.5
Current clamp or membrane potential (mV)
B
a
b
c
d
e
Sp 2
Sp 3
-80
-100
6.7
6.9
8.4
8.6
10.5
10.7
6.2
6.4
7.5
7.7
Sp 1
Sp 2
Sp 3
C
Aa
Ab
Bd
Ba
Be
Bb
Bc
0
-80
impose
current
clamp
2.0
4.0
6.0
8.0
10.0
Time (mins)

From Fig 5.6C, it is apparent that the membrane potential fluctuates greatly at very large current clamp potentials (eg. -130mV) as indicated by the excursions on the baseline, ie. it is difficult to current clamp these eggs at high potentials and it is even more difficult if the input resistance of the egg is low (eg. 100MΩ). The thicker baseline in Fig.5.6C compared to that in Fig.5.6A is also indicative of the larger fluctuations at high current clamp potentials (observed by Igusa & Miyazaki, 1983)

Figures 5.5 and 5.6 illustrated results from eggs which had a low membrane potential but were current clamped at high potentials before insemination. Figure 5.7 is an example of an egg bathed in Ca57K5, which was inseminated at the egg's membrane potential ie. no current was being injected in order to clamp the egg (the composition of Ca57K5 is the same as normal solution, with the following exceptions: $CaCl_2$, 57mM; NaCl, 40 mM) The whole time course of the experiment is shown in Fig 5.7C, the labelling of which refers to other parts of the figure. In this example "fertilization responses" were recorded at a low membrane potential and a high current clamp potential, in the same egg. At 2.2 minutes one sperm fused with the egg. No electrical event was observed in synchrony with this fusion (the membrane potential of the egg at the instant of fusion being -22mV). The instant of this fusion is indicated by the first arrow in Fig.5.7C. At 2.6 minutes a trh was recorded and a

second trh occurred before the first had repolarized to its initial potential (Fig.5.7Aa and 5.7C). A third trh was subsequently recorded at 4.4 minutes (Fig.5.7Ab and 5.7C). Then about 5.3 minutes after sperm addition the egg was current clamped to a potential of -106mV (as indicated in Fig.5.7C). When at this current clamp potential, a second sperm fused with the egg, causing an fsd at 6.2 minutes (indicated by the second arrow in Fig.5.7Cc, and the oscilloscope picture 5.7Bd). This was followed by a trd at 6.7 minutes (5.7 Ba). The third and final sperm fusion observed in this egg occurred at 7.6 minutes. Once again this was associated with an fsd which unlike that accompanying the second fusion appeared to have a plateau, (indicated by the third arrow in Fig.5.7C and the oscilloscope picture 5.7Be). This was a plateau in the sense that the potential had not quite returned to the initial current clamp potential of -106mV, even at the end of the experiment (about 4 minutes after this third fusion). At 8.4 minutes and 10.5 minutes, two further trds were recorded (shown in Fig.5.7C and in the oscilloscope pictures 5.7Bb and 5.7Bc respectively).

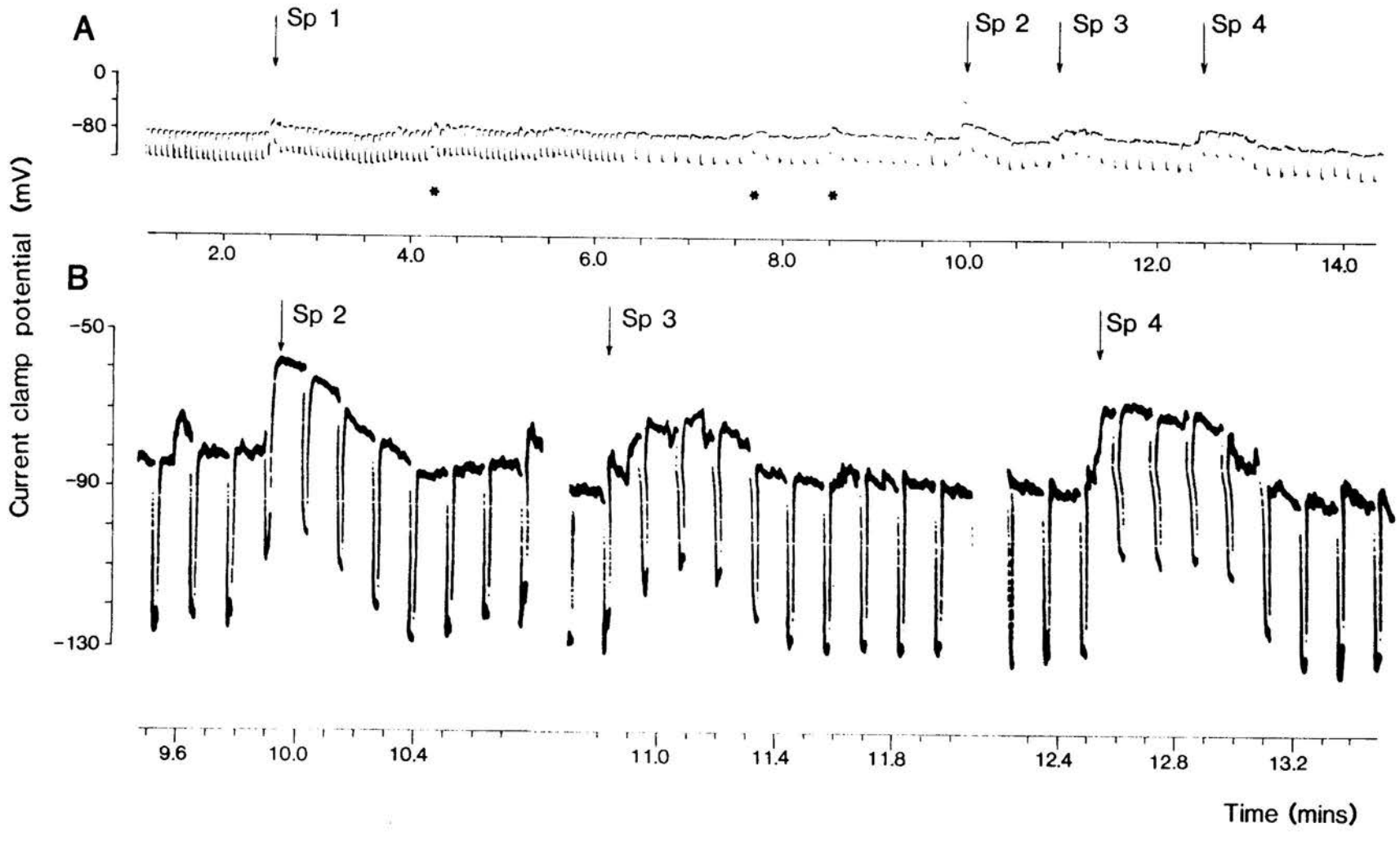
In Fig.5.7 the electrical responses observed in this egg have been classified into two groups. Firstly the slow responses which are the trhs and trds, and secondly the fast responses, which were those accompanying sperm fusions. As described above sperm fusions elicited only fsds and these were only when the

egg was current clamped at a high potential. When the egg was at a low membrane potential no electrical event was seen to accompany sperm fusion. In Fig.5.7A are displayed the electrical events which were observed at low potential (in this case -22mV). At this potential no fast responses were observed. In Fig.5.7B are shown the electrical events observed at high potential (-106mV). At high potential only trds and fsds were observed, whereas at low potential only trhs were observed.

It was not always possible to distinguish fsds from trds. One technique employed to help in this respect was continuous monitoring of the resistance by passage of constant current pulses at intervals of 3-5 secs. Duration of each pulse was fixed at 0.5-1.0 secs and the size of the pulse fixed at 0.1 to 0.5nA usually in the hyperpolarizing direction (see 2.3a). But unfortunately this had the effect of "distorting" the electrical response observed and in some cases maybe even "masking" it, particularly if the response was short lasting. One such recording is shown in Fig.5.8. This egg bathed in Ca4K5 had a membrane potential and an input resistance of -56mV and 260MΩ respectively. The egg was current clamped to a potential of -94mV prior to insemination. The egg was subsequently fertilized by four sperm, the times of the fusions being 2.4, 9.8, 10.8 and 12.4 minutes respectively. Each fusion is indicated by an arrow in Fig.5.8A. The final three fusions are shown in Fig.5.8B as

Figure 5.8

A zona-free hamster egg current clamped (initially) at -94mV fertilized by four hamster sperm. The timing of the fusion is indicated by arrows on the pen trace A. Oscilloscope pictures of the three final fusions are shown in B. Three further depolarizations with increases in conductance were observed at 4.2, 7.6 and 8.5 minutes. These were not associated with sperm fusions and have been indicated in A by asterisks. The egg was bathed in Ca4K5 and was confirmed by subsequent histology to be tetraspermic. At 6.3 minutes the frequency of pulses were reduced from 0.25 to 0.12Hz. Throughout this experiment the pulses were 0.2nA and 1 second.


A
Sp 1
Sp 2
Sp 3
Sp 4
0
-80
2.0
4.0
6.0
8.0
10.0
12.0
14.0
B
Sp 2
Sp 3
Sp 4
-50
-90
-130
9.6
10.0
10.4
11.0
11.4
11.8
12.4
12.8
13.2
Current clamp potential (mV)
Time (mins)

oscilloscope pictures. The reversal potentials (see Section C of this chapter) of these four fusion events were +105, +103, +144 and +101mV respectively. These reversal potentials were calculated in a similar manner to that of trhs, discussed earlier in this chapter. The values for the reversal potentials of these fusion events, given above, can only be considered to be crude approximations, since there is a large error involved in the calculation (see below).

Three other small depolarizations were observed, each with a rise in conductance (marked with asterisks in Fig.5.8A). These were not associated with sperm fusions. The reversal potentials of these three depolarizations were -21, -62 and -23mV respectively. It is therefore possible that the slow depolarization observed at about 7.6minutes (i.e. the one with the reversal potential of -61mV may be a trd response). As will be discussed in Section C of this chapter the mean reversal potential of trhs and trds in Ca4K5 is about -73mV. The other two depolarizations observed at 4.2 and 8.5 minutes may be an electrical indication of other events triggered by the first sperm fusion. But it is clear from this record that trhs or trds will be very difficult to "pick out" if the egg is current clamped at a potential close to the reversal potential of these responses, i.e. -73mV in Ca4K5. But measurements of the reversal potentials allow one to discriminate between fsds and any other

depolarizations, e.g. trds, because the reversal potentials of these two groups of responses are so well separated. Since the potential change at the peak of these fsds is small (that of the second, third and fourth fusion in the above example was 23, 17 and 22mV respectively) and the conductance increase also very small, there is likely to be an appreciable error in the calculation of the reversal potential. For example the fourth fusion in the above example had a reversal potential of +101mV. This was calculated as follows:-

P = size of electrotonic potential at rest = 43.

p = size of electrotonic potential during the response = 38.

v = amplitude of response = +22mV

e = reversal potential

E = current clamp or membrane potential = -88mV

$$\text{Since } e = \left(\frac{P}{P-p}\right) v + E$$

$$\text{Hence } e = \left(\frac{43}{43-38}\right)(+22) + (-88)$$

$$= \left(\frac{43}{5}\right)(+22) + (-88)$$

$$= +101\text{mV}$$

Assuming a maximum error possible in the measurement of P, p, v and E to be $\pm$1mV, then the maximum possible reversal potential

$$= \left(\frac{44}{4}\right)(+23) + (-87) = +166\text{mV}$$

Similarly the minimum possible reversal potential

$$= \left(\frac{42}{6}\right)(+21) + (-89) = +58\text{mV}$$

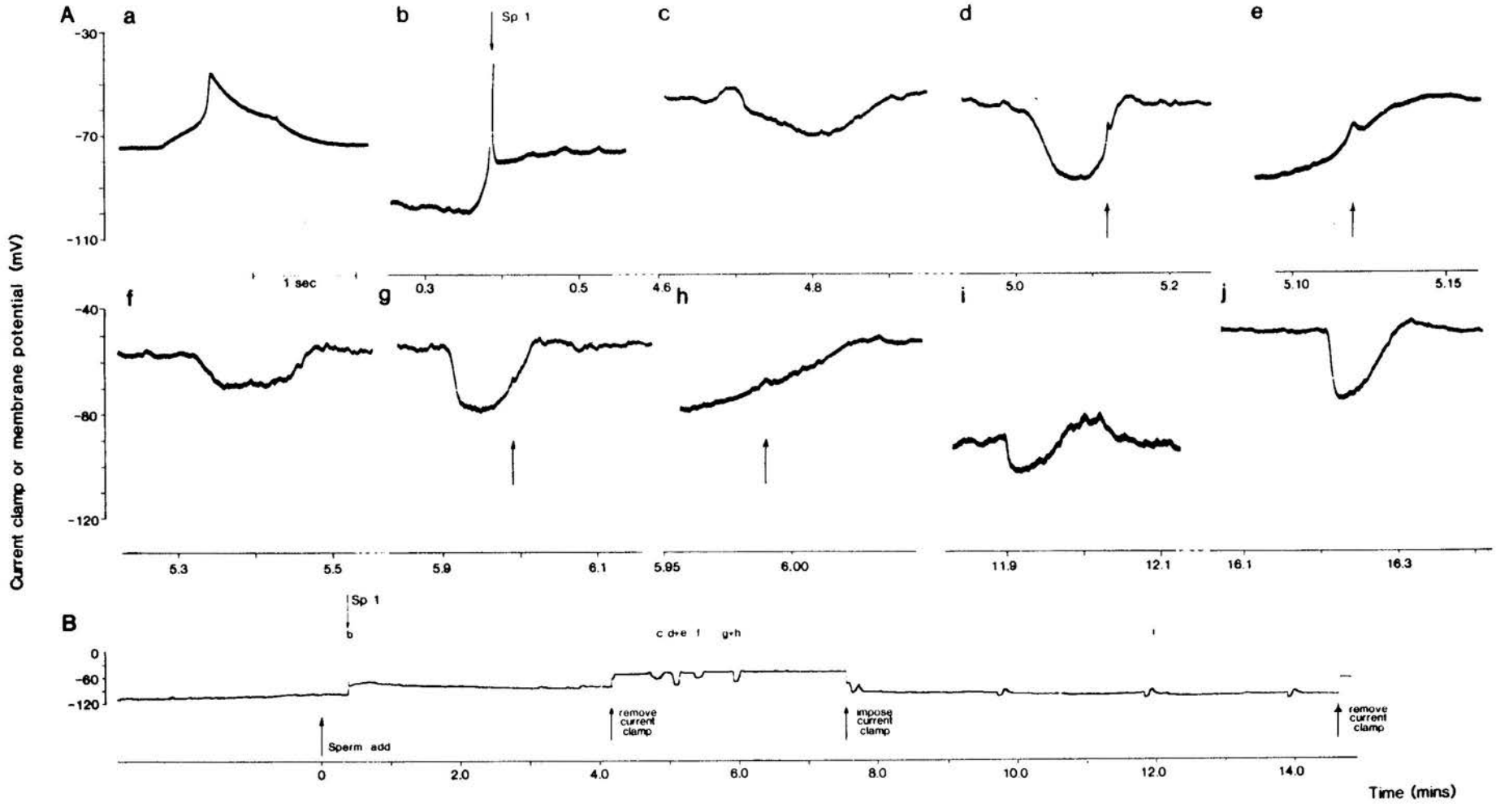
Therefore the two possible extremes for the reversal potential in the example taken are +58mV and +166mV. It is unlikely that in any one example all four parameters (P, p, v and E) were over or underestimated, so as to give the minimum or maximum possible result for the reversal potential. In any case the minimum possible reversal potential for the above example (+58mV) is clearly different from the reversal potential of trhs or trds (found to be -73mV see Section C of this chapter).

5.4 Homologous fertilizations of two high membrane potential hamster eggs

In this study, three eggs bathed in Ca4K5 were found to have high membrane potentials. Two of these were fertilized on insemination with hamster sperm and are discussed below (the impalement record for one of these is displayed in Figure 4.1D). The third was fertilized by mice sperm and is discussed in 5.5. On impalement the first of these two eggs had a membrane potential and an input resistance of -75mV and 250MΩ. At this potential electrically evoked action potentials were observed on passage of depolarizing pulses through the electrode. One such example is shown in Fig.5.9Aa.

Figure 5.9

Fertilization of a zona-free hamster egg which had a membrane potential and input resistance of -75mV and 250MΩ respectively. This egg was bathed in Ca4K5. An example of an electrically evoked action potential is shown in Aa. Prior to insemination the egg was current clamped at -100mV. Ab - Aj are oscilloscope pictures of various electrical responses recorded in this egg after monospermic fertilization. Fusion of the sperm with this egg caused an action potential shown in Ab. Current clamp was removed at 4.2 minutes, imposed again at 7.5 minutes, and then finally removed at 14.6 minutes. These procedures are indicated by arrows below the pen trace displayed in B. B also shows the instant of sperm addition, and a three minute recording of the current clamp potential prior to insemination. Letters above the pen trace in B, indicate that each response so marked on the pen trace is displayed in the form of an oscilloscope picture in A, with the corresponding letter. The instant of sperm fusion is indicated by an arrow above the pen trace in B and also in Ab. Arrows below the traces in Ad, Ae, Ag and Ah refer to small depolarizations which may be an indication of a calcium influx, like in anode break responses.


A
a
b
c
d
e
Sp 1
-30
-70
-110
1 sec
0.3
0.5
4.6
4.8
5.0
5.2
5.10
5.15
f
g
h
i
j
-40
-80
-120
5.3
5.5
5.9
6.1
5.95
6.00
11.9
12.1
16.1
16.3
Current clamp or membrane potential (mV)
B
Sp 1
b
c d+e f g+h
0
-60
-120
Sperm add
remove current clamp
impose current clamp
remove current clamp
0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
Time (mins)

The peak and threshold of this electrically evoked action potential were -44 and -64mV respectively.

This egg was inseminated at zero time after current clamping it at a potential of about -100mV. Then 0.4 minutes later a sperm fusion elicited a sperm evoked action potential (shown in Fig.5.9Ab). The peak, the threshold and the plateau potential level of the sperm evoked action potential were -43, -82 and -70mV respectively. The plateau of this action potential gradually hyperpolarized and superimposed on it were two possible trds at 3.1 and 3.7 minutes. But because of their small amplitude, and the absence of a conductance measurement it is not possible to say with certainty, whether or not they are trds. Both these are shown in the pen trace displayed in Fig.5.9B.

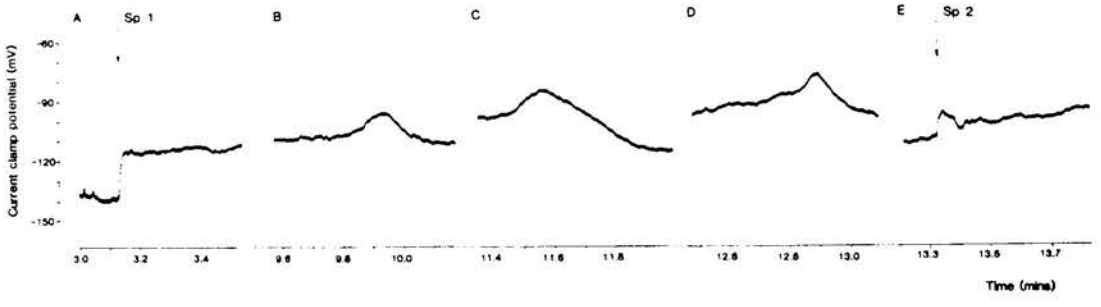
At about 4.2 minutes, the current clamp was removed so that the egg was held at its membrane potential, which was found to be -60mV. At this potential two trhs were observed (Fig.5.9Ad and 5.9Ag) at times 5.0 and 5.9 minutes. But curiously either side of the first of these trhs was a small hyperpolarization (Fig.5.9Ac and 5.9Af), occurring at 4.7 and 5.3 minutes respectively. The amplitudes and durations of which were 15mV, 12secs and 12mV, 9.6secs respectively. Whereas the amplitude and duration of the two trhs were 26mV, 9 secs and 24mV, 7.2 secs. These small hyperpolarizations may be a different type of response to trhs or they are just an indication of the variability of trhs.

Usually the downward stroke or the hyperpolarizing phase of trhs is faster (i.e. rate of change of potential is greater in magnitude) than the upward or repolarizing phase. But in Fig.5.9Ad it is apparent that the converse applies. This may be as a result of the fact that the peak of the trh (-78mV in Fig.5.9Ad) is more hyperpolarized than the threshold for the electrically evoked action potential. Hence during the upstroke of the trh because the membrane potential "passes through" the threshold for the electrically evoked action potential (-64mV), a response which is reminiscent of the anode break response is observed (marked by arrows below the oscilloscope traces). Indeed, very small depolarizing excursions are seen in the upstroke of the trhs shown in Fig.5.9Ad and 5.9Ag (more prominent in the former, indicated by arrows below the two oscilloscope pictures). Both these trhs, (in Fig.5.9Ad and Fig.5.9Ag) are shown at a faster sweep speed in Fig.5.9Ae and 5.9Ah respectively.

At 7.5 minutes current clamp was again imposed on the egg, such that the egg was clamped at -105mV. Immediately a response was observed followed by three others of a similar nature at times 9.7, 11.8 and 13.9 minutes. These responses have been interpreted as being trds with preceding hyperpolarizations and will be discussed in Section C of this chapter. The one observed at 11.9 minutes is shown in the form of an oscilloscope picture in 5.9Ai (all of them are

Figure 5.10

A zona-free hamster egg with a membrane potential and an input resistance of -100mV and 200MΩ respectively was fertilized by two sperm. Each fusion is indicated by an arrow above the oscilloscope picture in A and E. B - D are oscilloscope pictures of the three trds observed after the first fusion. The egg was bathed in Ca4K5 and was confirmed by subsequent histology to be dispermic.


A
So 1
B
C
D
E
So 2
Current clamp potential (mV)
-60
-90
-120
-150
3.0
3.2
3.4
9.8
9.8
10.0
11.4
11.6
11.8
12.6
12.8
13.0
13.3
13.5
13.7
Time (mins)

displayed in the pen trace, 5.9B).

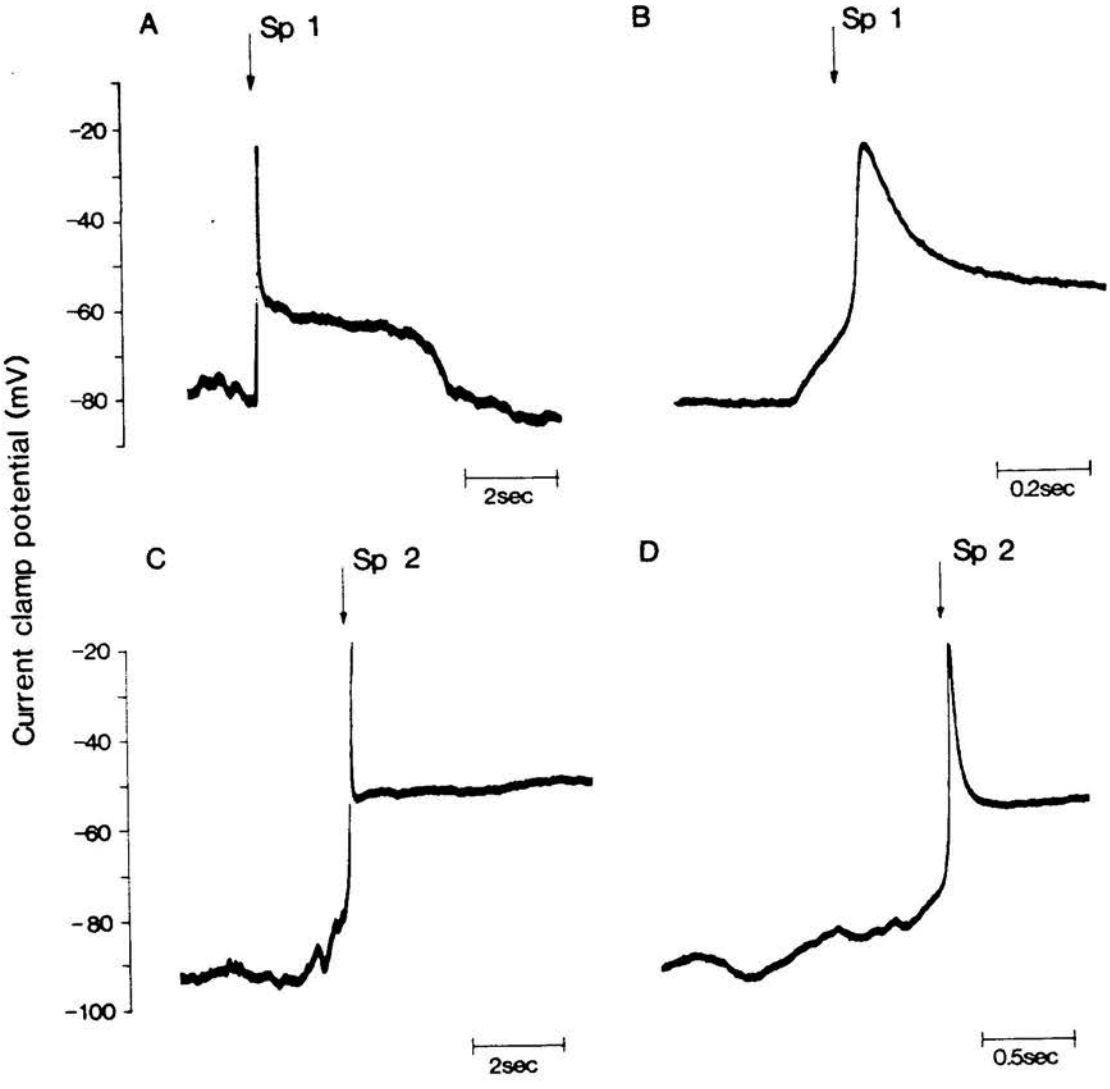
At 14.6 minutes the current clamp is again removed. This was followed by five trhs (observed at the egg's membrane potential of -49mV) at times 16.2, 18.3, 19.7, 20.8 and 22.0 minutes. None of these is shown on the pen trace (Fig.5.9B). But the first of these is shown in 5.9Aj in which an after depolarization was observed. The reversal potentials of the responses at 18.3, 19.7, 20.8 and 22.0 minutes were -84, -92, -84 and -88mV respectively.

In summary this egg was fertilized by one sperm only, the action potential elicited at the instant of fusion, had a plateau which had not returned to its initial current clamp potential of -100mV, 3.8 minutes later (at which point the current clamp was removed). Small hyperpolarizations were observed (which were not trhs) and also trds with preceding hyperpolarizations.

The second high potential egg fertilized with hamster sperm had a membrane potential and an input resistance of -100mV and 200MΩ. The threshold and the spike peak for the electrically evoked action potential in this egg was -60mV and -19mV respectively. But the fluctuations in it s membrane potential were large. Nevertheless it was fertilized by two sperm (after it had been current clamped at a potential of about -140mV), and each fusion was accompanied by an fsd (Fig.5.10A and 5.10E). After the first fusion three trds were recorded. These are shown in Fig.5.10B-5.10D. But because of the fluctuations in the membrane

Figure 5.11

Oscilloscope pictures of action potentials recorded in zona-free hamster eggs fertilized by mice sperm. A is a sperm evoked action potential recorded in an egg bathed in Ca4K5. No other electrical events were recorded in this egg. Histology showed that this egg had been fertilized by one sperm. The same action potential is displayed at a faster sweep speed in B. No predepolarization events were observed in this case. C shows another sperm evoked action potential during fertilization of another hamster egg by mouse sperm. Prior to this action potential one sperm had already fused with the egg (this event having been seen electrically as an fsd). The second fusion elicited this action potential shown in C and D (D is the same action potential as in C but at a faster sweep speed). Predepolarization events were noted prior to this action potential. The plateau of this action potential did not return to the initial current clamp potential. The arrows above the oscilloscope pictures show that these action potentials were accompanied by sperm fusions.


A
Sp 1
B
Sp 1
-20
-40
-60
-80
Current clamp potential (mV)
2sec
0.2sec
C
Sp 2
D
Sp 2
-20
-40
-60
-80
-100
2sec
0.5sec

potential of this egg, it was not possible to measure durations and amplitudes of these reponses accurately. Histology confirmed this egg to be dispermic.

5.5 Heterologous fertilizations of zona-free hamster eggs with mouse sperm (Table 5.1)

Five hamster eggs were fertilized with mouse sperm. One of these eggs bathed in Ca2K5 gave no electrical response during recording but was found subsequently by histology to be dispermic.

Another egg bathed in Ca4K5 was fertilized by one mouse sperm. This sperm fusion elicited the action potential shown in Fig.5.11A. The same action potential is shown at a faster sweep speed in Fig5.11B. Recording was continued for a further 270secs after the end of this action potential, during which time no further electrical event was recorded.

In two other hamster eggs bathed in Ca4K5 fsds were observed to accompany sperm fusion. One of these eggs was monospermic and the other dispermic.

In the fifth egg, the first fusion was correlated with an fsd whereas the second fusion was accompanied by an action potential. This action potential is shown in Fig.5.11C and also in 5.11D (at a faster sweep speed). This egg also bathed in Ca4K5 actually had a high membrane potential of -70mV (no measurement of its input resistance was made), but was nevertheless current clamped to a potential of -107mV, prior to

Table 5.1

The results of heterologous fertilizations of zona-free hamster eggs with mouse sperm. The table shows the electrical events accompanying five such fertilizations. The bathing solution and the results of histology for each egg are also shown.

Bathing Solution	FSD			SPERM EVOKED ACTION POTENTIALS				NO ELECTRICAL EVENT	HISTOLOGY
	Peak potential (mV)	Amplitude (mV)	Duration (sec)	Spike peak (mV)	Threshold (mV)	Duration (sec)	Plateau potential level (mV)		
Ca2K5								✓	Dispermic
Ca4K5				-24	-64	8	-64		Monospermic
Ca4K4	-104	6	9						Monospermic
Ca4K5	-80 -74	10 14	42 16						Dispermic
Ca4K5	-70	14	11	-17	-70	plateau did not return	-52		Dispermic

TABLE 5.1

insemination. Although the current clamp potential was lower at the time of the second fusion (about -94mV).

The other four eggs described above were also current clamped at high potentials prior to insemination with mouse sperm.

Interestingly, in all five fertilizations, no trds were observed. No experiments were performed in which eggs were maintained at low potentials prior to insemination. Hence one would not have expected to see trhs, since in all five experiments described above the eggs were current clamped at potentials in the region of -100mV.

The sperm evoked action potential illustrated in Fig.5.11C and 5.11D has a predepolarization like those seen during homologous fertilization of hamster eggs.

Table 5.1 summarizes the electrical events recorded during heterologous fertilization of hamster eggs with mice sperm.

SECTION B

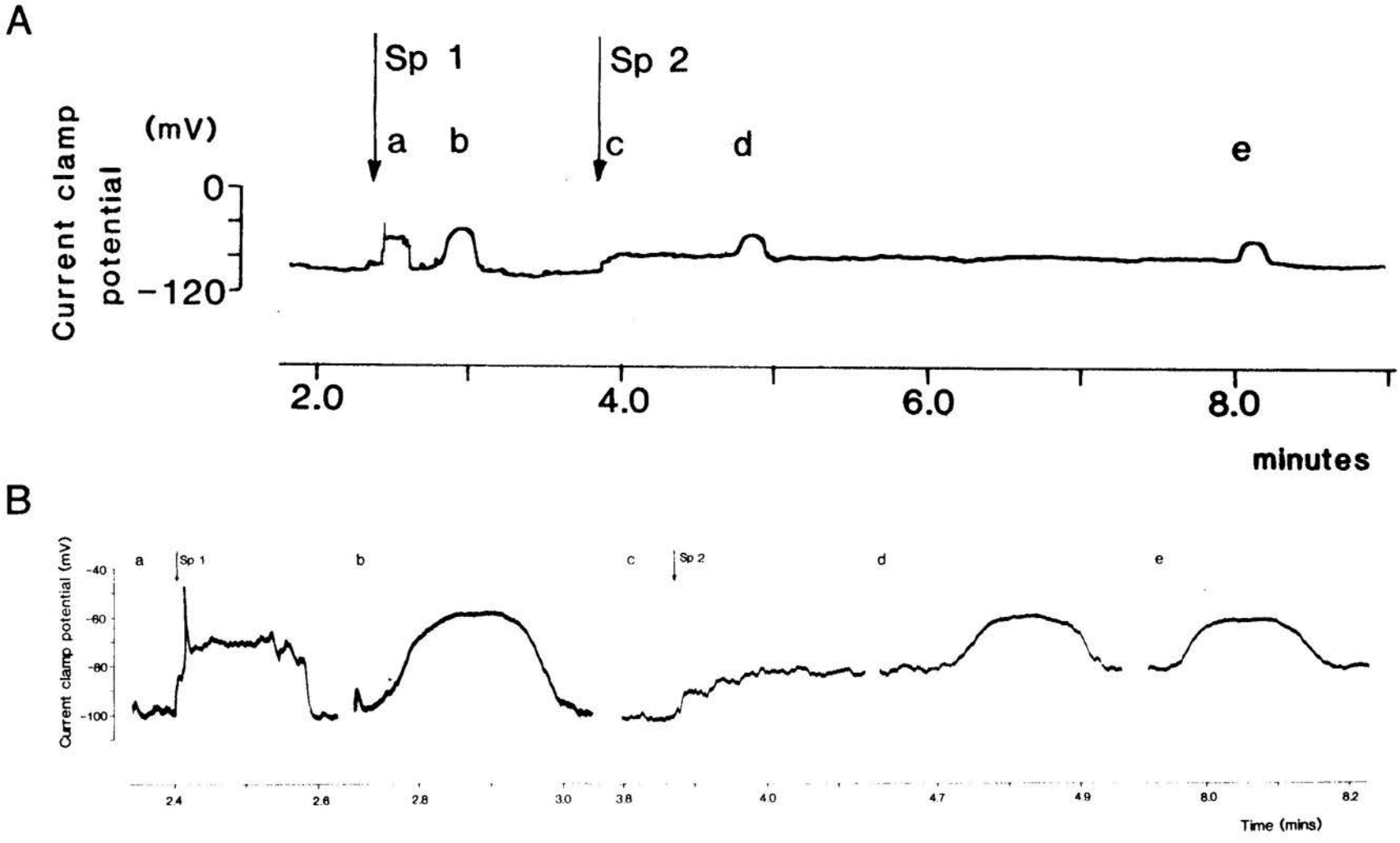
FERTILIZATIONS IN HIGH POTASSIUM SOLUTIONS

5.6 Homologous fertilizations of low potential hamster eggs current clamped at high potentials prior to insemination

Fig.5.12A illustrates a dispermic fertilization in an egg bathed in Ca2K25. The egg was current clamped at -100mV prior to insemination although the current

Figure 5.12

Homologous fertilization of a zona-free hamster egg bathed in Ca2K25 and current clamped at a potential of -100mV (the current clamp potential is -98mV just prior to the first fusion shown in Ba). A is a pen trace recording obtained during this experiment. The arrows above the pen trace indicate the times of sperm fusions. The letters above individual events shown in A correspond to the labelling given to the oscilloscope picture of that event shown in B. The first fusion in this egg elicited an action potential (peak = -47mV, threshold = -68mV, plateau potential level = -68mV and duration = 11.2 seconds) shown in Ba. The second fusion was accompanied by an fsd, shown in Bc. Subsequent histology confirmed this egg to be dispermic. A predepolarization occurred prior to the sperm evoked action potential which accompanied the first fusion (A).

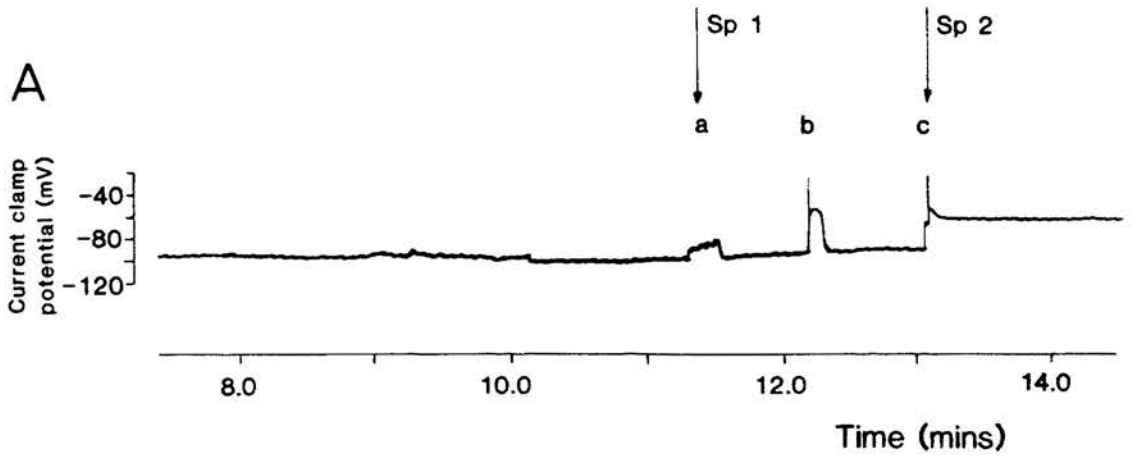

A
Sp 1
Sp 2
a
b
c
d
e
(mV)
0
−120
Current clamp potential
2.0
4.0
6.0
8.0
minutes
B
Current clamp potential (mV)
-40
-60
-80
-100
a
Sp 1
b
c
Sp 2
d
e
2.4
2.6
2.8
3.0
3.8
4.0
4.7
4.9
8.0
8.2
Time (mins)

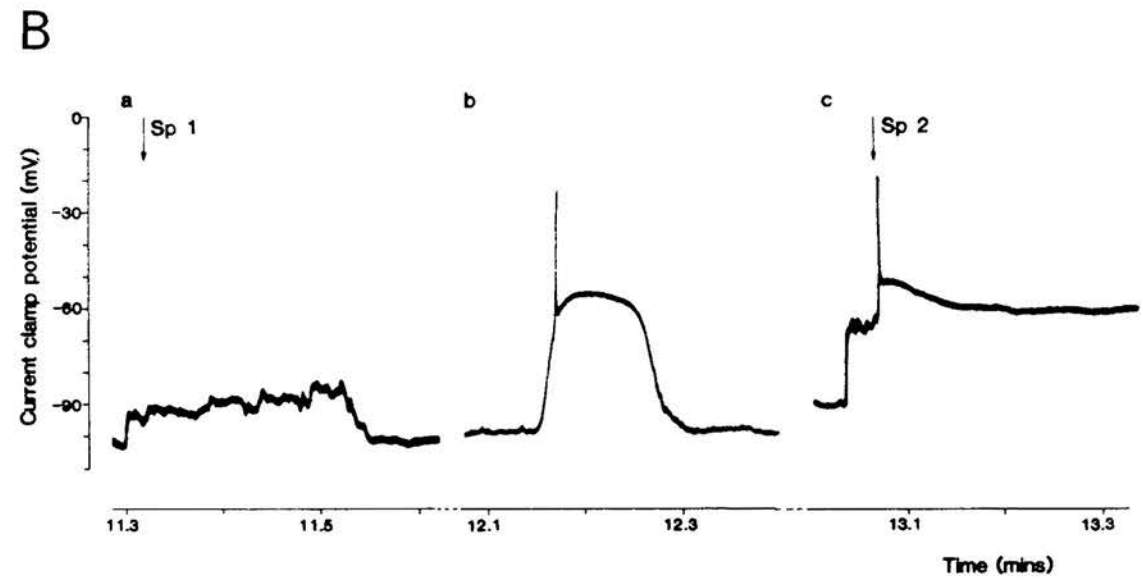
clamp potential was -98mV just prior to the first fusion. The first sperm fusion at 2.4 minutes elicited an action potential shown in Fig.5.12Ba. This was followed at 2.7 minutes by trd shown in Fig.5.12Bb. The peak potential of the trd was -57mV and its duration was 16 seconds. The trds observed in the experiments illustrated in Section A of this chapter, always had peak potentials which were more hyperpolarized than about -80mV. The reason for the more depolarized peak potential in this experiment is that the egg is bathed in a solution containing a five fold greater concentration of potassium (i.e. 25mM as opposed to 5mM in all the experiments in Section A). Since trhs and trds are caused by a calcium activated potassium conductance (Miyazaki & Igusa, 1982), they are therefore dependent on the external potassium concentration. Hence in a medium containing a higher concentration of potassium, the reversal potentials for these responses will be more depolarized, and hence that of the peak potentials (all other parameters being equal). Miyazaki & Igusa (1982) obtained a mean reversal potential of -82.7mV, in a solution containing 5.5 mM potassium. Hence in a solution containing 25mM potassium, the expected reversal potential is calculated to be -43.2mV (i.e. -82.7--60 [log 25/5.5]).

A second fusion was observed in this egg, which was seen electrically as an fsd, shown in Fig.5.12Bc. But this fsd (peak potential = -82mV and amplitude

Figure 5.13

A dispermic fertilization of a zona-free hamster egg bathed in Ca2K25. A is a pen trace recording obtained during the experiment. Arrows in A indicate the times of the sperm fusion. The letters above the electrical events shown in A refer to the corresponding oscilloscope pictures in B. The first fusion evoked an fsd (Ba) and the second fusion an action potential (Bc). This action potential had a maintained plateau. Confirmatory evidence of dispermy was obtained by histology.


A
Sp 1
Sp 2
a
b
c
Current clamp potential (mV)
-40
-80
-120
8.0
10.0
12.0
14.0
Time (mins)

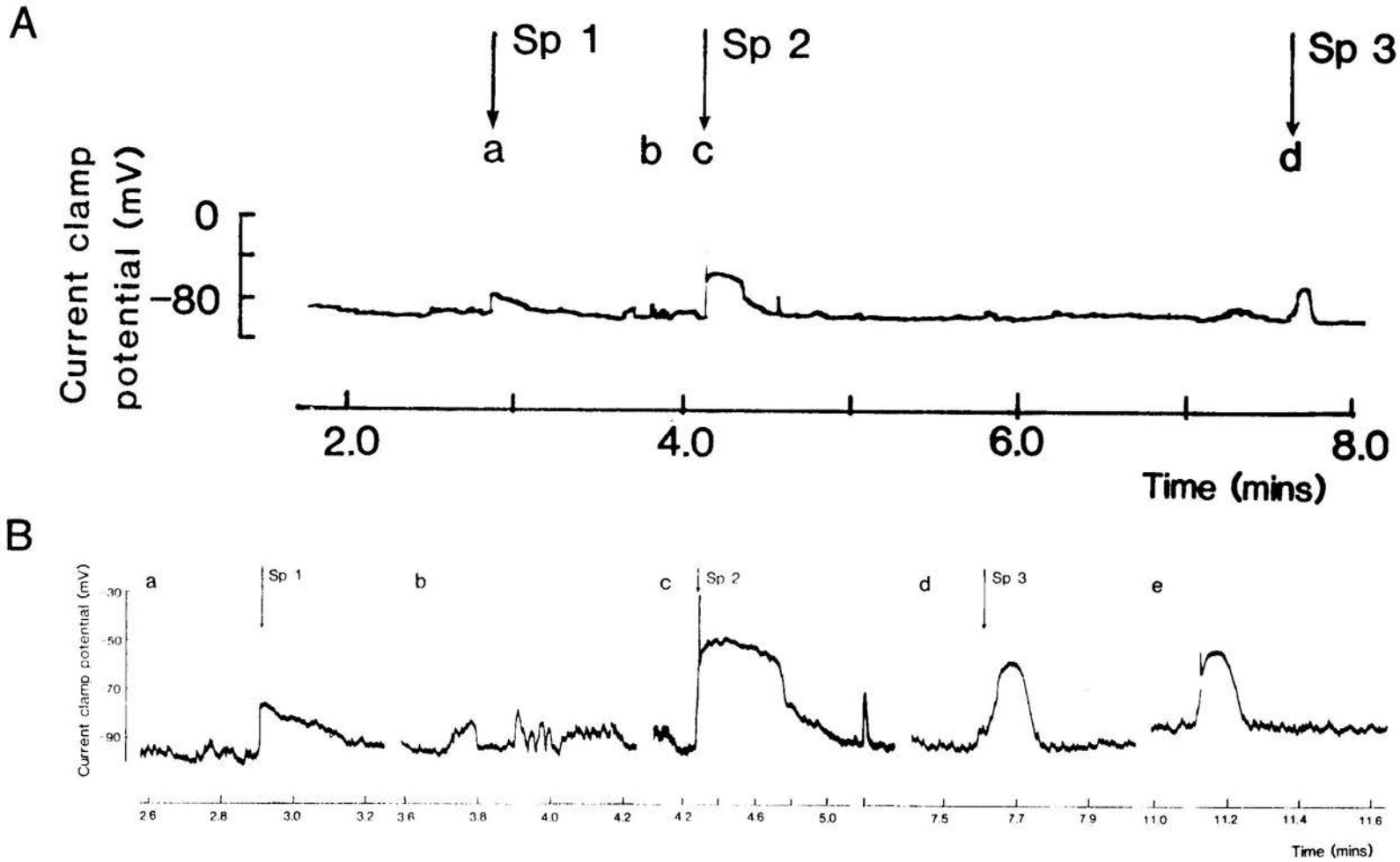

B
a
b
c
Sp 1
Sp 2
Current clamp potential (mV)
0
-30
-60
-90
11.3
11.5
12.1
12.3
13.1
13.3
Time (mins)

= 18mV), appeared to plateau at its peak potential. This potential did not return to its initial current clamp potential (i.e. about -100mV). Superimposed on this plateau were two further trds at times 4.7 (Fig.5.12Bd) and 8.0 (Fig.5.12Be) minutes. The peak potentials and durations were -60mV, 12 secs and -62mV, 13 secs. This egg was subsequently confirmed by histology to be dispermic.

A feature which the next record illustrates (Fig.5.13) is that trds in high potassium solutions can themselves give rise to action potentials. Such responses have been called trd spikes in this study. The reason they arise is that in high potassium solution the potassium reversal potential is more depolarized than the threshold for the electrically evoked action potential. Hence if the peak potential of a trd is more depolarized than the threshold for the electrically evoked action potential, then a spike will be elicited by the depolarizing phase of a trd. Although in the example shown in Fig.5.13, it is possible to distinguish between a trd spike and a sperm evoked action potential, this was not always the case. The fertilization responses illustrated in Fig.5.13A were recorded in an egg bathed in Ca2K25, which was fertilized by two sperm. The egg was current clamped at a potential of -107mV before adding sperm to the chamber. The first sperm fusion gave rise to an fsd (at which time the current clamp potential was -104mV) which is shown in Fig.5.13Ba, at 11.3 minutes. At 12.2

Figure 5.14

A zona-free hamster egg current clamped at -96mV bathed in Ca2K25 and fertilized by three sperm. A is a pen trace recording obtained during the experiment, the arrows indicating the times of the three fusions. The letters above the electrical events shown in the pen trace refer to corresponding oscilloscope pictures in B. Ba, Bc and Bd are the electrical events corresponding to the three sperm fusions. Bb are events which have been interpreted as being "fusion events" following the first sperm fusion or possibly even predepolarization events associated with the second fusion. Histology confirmed this egg to be trispermic.


A
Sp 1
Sp 2
Sp 3
a
b
c
d
Current clamp potential (mV)
0
-80
2.0
4.0
6.0
8.0
Time (mins)
B
a
Sp 1
b
c
Sp 2
d
Sp 3
e
Current clamp potential (mV)
-30
-50
-70
-90
2.6
2.8
3.0
3.2
3.6
3.8
4.0
4.2
4.2
4.6
5.0
7.5
7.7
7.9
11.0
11.2
11.4
11.6
Time (mins)

minutes a trd spike was recorded (Fig.5.13Bb). A second sperm fusion was recorded at 13.1 minutes, but this elicited an action potential (Fig.5.13Bc) which had a plateau (peak = -24mV, threshold -68mV and plateau potential level = -70mV). In another four minutes of recording this plateau did not return to its initial current clamp potential. Indeed at 16.4 minutes another trd spike was noted. Since at that time hyperpolarizing current pulses were being used to monitor any conductance changes, it was possible to calculate the reversal potential of this last trd spike (not shown in Fig.5.13). The reversal potential was found to be -58mV (this is the reversal potential of the trd "portion" of the trd spike response). There is a small depolarization (amplitude 5mV, duration 49secs) at 9.2 minutes (shown on pen trace in Fig.5.13A) but at this instant no sperm were seen to be straight or immotile on the egg.

Another example of a fertilization in Ca2K25 is shown in Fig.5.14. This particular egg was also inseminated after current clamping it at a high potential (-98mV). The arrows above the pen trace in Fig.5.14A show the times of the three fusions. The electrical events caused by fertilization have also been displayed in Fig.5.14B as oscilloscope pictures. The first sperm fusion elicited in fsd (5.14Ba). This was followed by brief depolarizing excursions, shown in Fig.5.14Bb. These were not associated with sperm

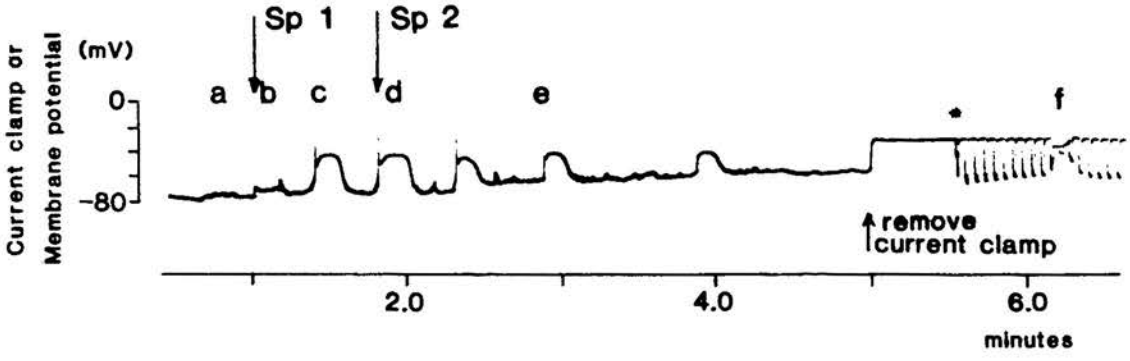
fusions, but were presumably events triggered off by the first fusion. Such events were also seen in Fig.5.5A of this chapter. Then at 4.2 minutes a second sperm fused with the egg causing this time an action potential which is shown in Fig.5.14Bc (peak = -33mV, threshold = -76mV, duration = 24secs, plateau potential = -52mV). It is possible that some of the depolarizing excursions shown in Fig.5.14Bb may be linked to this second fusion. The third fusion occurred at 7.6 minutes and this elicited a trd which seemed to be merged with a predepolarization (Fig.5.14Bd). Then finally a trd spike was recorded at 11.1 minutes. In this egg three fusions gave three different electrical responses. The third fusion shown in Fig.5.14Bd caused a trd with a predepolarization (peak potential of the trd = -60mV). The peak potential of the trd spike later observed in this egg was -56mV. It is curious that the trd accompanying the third fusion was not also a trd spike.

Figure 5.15 lends support to the idea that a trd, trh or a trd spike which is closely associated with a sperm fusion is longer in duration than a similar response not associated with a sperm fusion. The first sperm fusion elicited an fsd (amplitude = 9mV, duration = 12secs) at 1.0 minute. This was followed by a trd spike at 1.4 minutes (peak = -44mV, duration = 14 secs, spike peak = -35mV). The second sperm fusion was in synchrony with another trd spike at 1.8 minutes (peak = -40mV, duration = 16 secs, spike peak = -24mV).

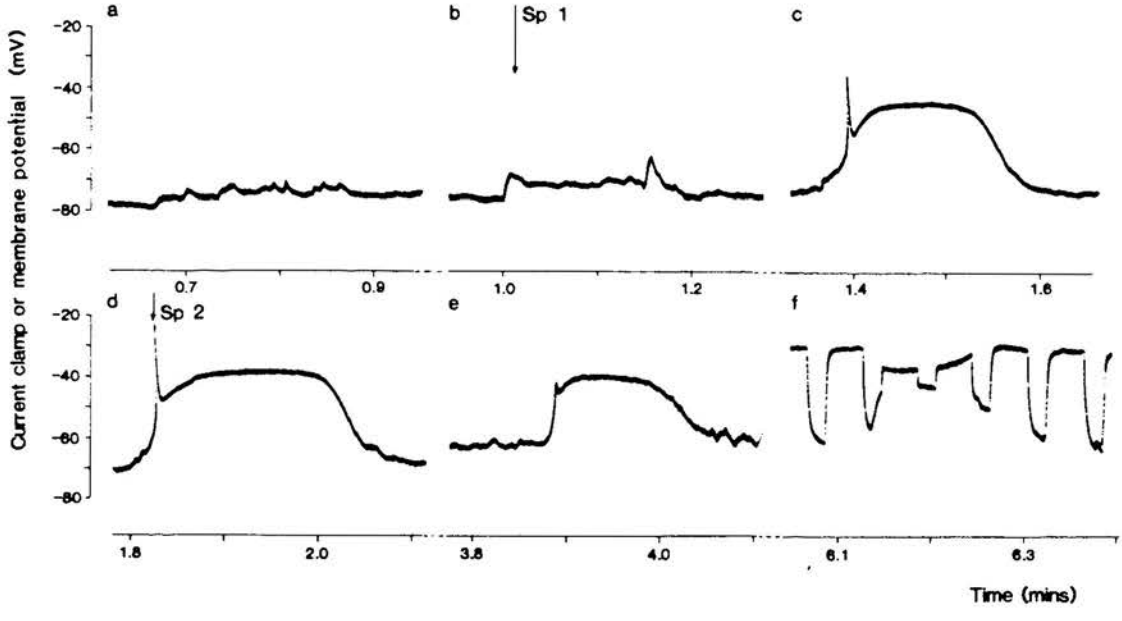
Figure 5.15

A. Pen trace of electrical events observed during fertilization of a zona-free hamster egg bathed in Ca2K25. The egg was fertilized by two sperm, each fusion is indicated by arrows above the trace. The letters above the trace refer to corresponding oscilloscope pictures in B. The first fusion is accompanied by an fsd (Bb) and the second by a trd spike (Bd). The arrow below the trace in A at 5.0 minutes indicates the time at which current clamp was removed, and so the egg was at its membrane potential (-30mV). The asterisk above the pen trace indicates the time at which hyperpolarizing current pulses were passed in order to monitor conductance changes (0.2nA, 1 second, 0.30Hz).

A


Sp 1
Sp 2
(mV)
0
-80
Current clamp or
Membrane potential
a
b
c
d
e
f
remove
current clamp
2.0
4.0
6.0
minutes

B


a
b
Sp 1
c
-20
-40
-60
-80
0.7
0.9
1.0
1.2
1.4
1.6
d
Sp 2
e
f
Current clamp or membrane potential (mV)
1.8
2.0
3.8
4.0
6.1
6.3
Time (mins)

Subsequently two more trd spikes and a trd were noted at times 2.3, 2.9 and 3.9 minutes respectively. Then at 5.0 minutes the current clamp was removed, such that the egg was at its membrane potential, which in this case was -30mV. At this potential a trh was recorded (Fig.5.15Bf) the reversal potential of which was -38mV. But the result of particular interest in this experiment was that the trd spike which accompanied the second fusion (Fig.5.15Bd) was longer in duration than any of the other trd spikes or trds observed (duration of trd spike at 1.4 minutes = 14secs, of trd spike at 2.3 minutes = 10secs, of trd spike at 2.9 minutes = 10secs and of trd at 3.9 minutes = 10secs, whereas the duration of the trd spike accompanying the second fusion was 16secs).

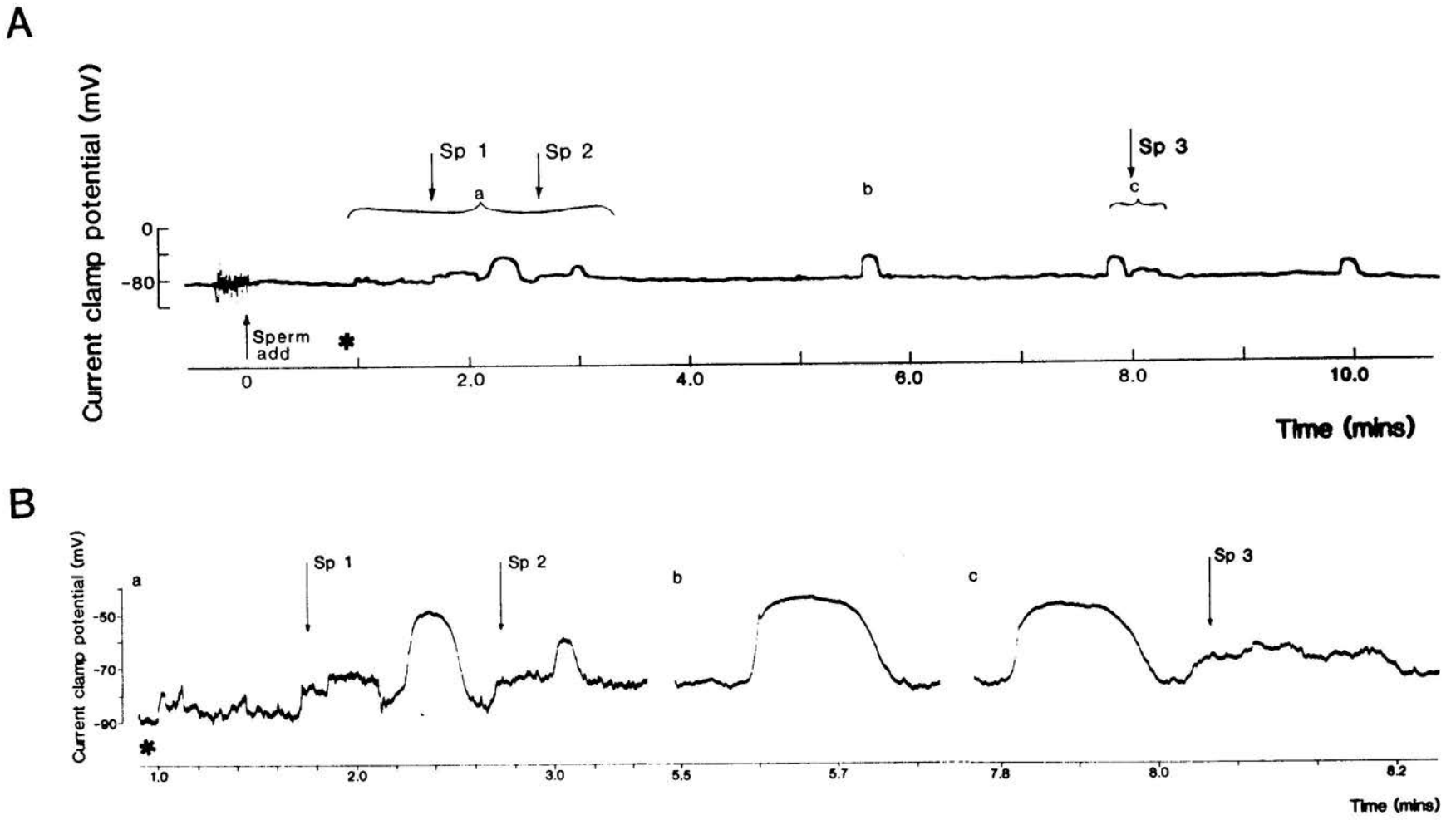
There was a depolarization at 0.7 minutes (amplitude = 7mV, duration = 12secs) shown in Fig.5.15Ba, but at this time no sperm were seen to be straight or immotile. Histology confirmed this egg to be dispermic.

5.7 Homologous fertilizations of low potential hamster eggs current clamped at high potentials prior to insemination in low sodium solutions

Two different low sodium solutions were used, but both were Ca2K25. In one kind all the sodium chloride had been replaced by lithium chloride and in the other the sodium chloride had been replaced by

Figure 5.16

A. Pen trace of the electrical events observed in an egg bathed in Ca2K25 (choline), when it was fertilized by three sperm. Prior to insemination the egg was current clamped -90mV. The first arrow below the pen trace shows the time of sperm addition. The asterisk below the pen trace indicates the instant of attachment of the first sperm. This sperm became straight and immotile, about 40 seconds later, as indicated by the first arrow above the pen trace. Two more fusions occurred in this egg, as marked by two more arrows above the pen trace. Letters above the trace in A refer to corresponding oscilloscope pictures in B. The arrows above the oscilloscope pictures and the asterisk below, have the same meaning as in A.


A
Current clamp potential (mV)
Sp 1
Sp 2
Sp 3
a
b
c
0
-80
Sperm add
0
2.0
4.0
6.0
8.0
10.0
Time (mins)
B
Current clamp potential (mV)
a
b
c
Sp 1
Sp 2
Sp 3
-50
-70
-90
1.0
2.0
3.0
5.5
5.7
7.8
8.0
8.2
Time (mins)

choline chloride. Therefore the only sodium present was in the form of sodium lactate, sodium pyruvate and sodium hydroxide (used to buffer the solution). Hence the total sodium in these low sodium solutions was about 24mM. An egg bathed in Ca2K25 (choline) was current clamped at -90mV, and then inseminated. It was fertilized by three sperm. Each fusion is indicated by an arrow above the trace or the oscilloscope picture in Fig.5.16A and B. The first sperm attached to this egg at the point marked by an asterisk, about 1 minute after insemination. But it did not become straight and immotile until about 40secs later, at which point an fsd was observed (Fig.5.16Ba). This was immediately followed by a trd at 2.2 minutes (Fig.5.16Ba). At the end of this trd a second sperm fused with the egg at 2.7 minutes which was observed electrically as another fsd. Superimposed on the plateau of this fsd was a small depolarization. Its peak potential was -62mV, whereas that of the trd observed at 2.2 minutes was -49mV.

At 5.6, 7.8 and 9.9 minutes, three further trds were observed, (the first two are displayed as oscilloscope pictures in Fig.5.16Bb and 5.16Cc respectively, all three are shown on the pen trace Fig.5.16A). The peak potentials of these last three trds were -45, -46, and -46mV respectively. These values are comparable with the peak potential of the first trd observed in this egg (-49mV). Therefore that small depolarization observed at 3.0 minutes (peak

Table 5.2

Membrane potentials and input resistances recorded in zona-free hamster eggs (bathed in various solutions at 34-36^{o}C), with intracellular microelectrodes. Some of these eggs were subsequently inseminated, a proportion of which were successfully fertilized. Ranges of membrane potentials and input resistances recorded before and after fertilization are noted in the last two columns (a positive value indicating a depolarization or an increase in resistance, and a negative value denoting a hyperpolarization or a decrease in resistance after fertilization). The results are shown as the mean $\pm$ SD (n=) wherever possible. Otherwise the actual values obtained are shown (when numbers were too small to allow statistical analysis), in italics. The results are tabulated in four groups - those in normal potassium, high potassium, very high potassium and low sodium solutions.

SOLUTION	Membrane potential mean ± SD (n=) (mV)	Input resistance mean ± SD (n=) (MΩ)	Range of membrane potentials (mV)	Range of input resistances (MΩ)	Difference between the membrane potential before & after fert^{n} mean ± SD (n=) (mV)	Difference between the input resistance before & after fert^{n} mean ± SD (n=) (MΩ)
Ca2K5	-26±8 (31)	150±70 (31)	-8 - -47	30 - 280	-2±10 (10)	
Ca4K5	-38±14 (56)	240±100 (55)	-19 - -100	80 - 400	+6±11 (21)	-160±100(7)
Ca12K5	-37	200			-5	
Ca42K5	-31±8 (9)	170±40 (9)	-22 - -47	100 - 250	+2±9 (6)	50 160
Ca57K5	-25 -30 -38 -43	120 150 160 200	-25 - -43	120 - 200	+2 +28	
Ca2K25	-34±8 (45)	190±60 (46)	-21 - -56	50 - 300	+7±8 (31)	+30 -30 -30 -60 -100 -150
Ca4K25	-37±9 (14)	230±90 (14)	-29 - -38	150 - 230	-4 0 0 +1	
Ca7K25	-38	300			+12	
Ca10K25	-33±10 (7)	250±80 (6)	-22 - -50	160 - 370	+14	
Ca20K25	-30	100				
Ca7K40	-24	200			+12	
Ca2K25 (choline)	-28±6 (5)	140±40 (5)	-27 - -39	80 - 200	0 +2 +8	-60
Ca2K25 (lithium)	-6± 2 (7)	50±10 (7)	-3 - -10	30 - 70	-5	

TABLE 5.2

potential = -62mV), is apparently some different form of response (which actually may be correlated with the small hyperpolarizations noted in Fig.5.9Ac and Fig.5.9Af).

The durations of the four trds mentioned above, and shown in Fig.5.16A were 21, 11, 11 and 10 secs respectively. Thus the duration of the first trd following the first fusion is almost twice that of the remaining three (note that the time scale in Fig.5.16Ba is different to that in 5.16Bb and 5.16Bc).

A third fusion occurred at 7.9 minutes which was also accompanied by an fsd (although it did not appear to be very fast) as shown in Fig.5.16Bc. Duration, amplitude and peak potential of this fsd were 17 secs, 11mV and -64mV respectively.

SECTION C

ANALYSIS OF RESULTS

5.8 Membrane potentials and input resistances of zona-free hamster eggs (Table 5.2)

Table 5.2 shows the membrane potentials and input resistances of hamster eggs bathed in the various solutions used throughout the course of these experiments. Most of the eggs described in Section A and Section B of this chapter were current clamped (usually in the range -80 to -120mV), before they were inseminated. The experiments described in 5.2 were performed on eggs which were not current clamped, i.e.

the eggs were inseminated and fertilized at the egg's membrane poential.

The membrane potential of the egg (whether it be clamped or the actual membrane potential) has been referred to as being low, if it was more depolarized than the threshold for the electrically evoked action potential (see Chapter 4) and the egg has been called a low potential egg. Similarily if the egg has a membrane potential more hyperpolarized than the threshold, the egg has a high potential and is referred to as being a high potential egg.

On impalement three eggs out of 56 bathed in Ca4K5 were found to have high membrane potentials. Two of these three eggs were fertilized by hamster sperm (5.4) and the third by mouse sperm (5.5). All other eggs scored in Table 5.2 had low membrane potentials. The problems involved during intracellular recording in hamster (and also mice) eggs and the purpose of current clamping low membrane potential eggs to high potentials prior to insemination has been discussed in Chapter 4.

All the impalements scored in Table 5.2 were performed whilst the egg was bathed in a solution at a temperature between 34 and 36^{o}C.

The membrane potentials and input resistances were higher in Ca4K5 compared to those in Ca2K5 ($p<0.01$: Fisher Behrens test). But both the membrane potentials and input resistances in high potassium solutions were not significantly different in solutions with calcium

concentrations of 2mM and 4mM ($p>0.05$: two sample t test and Fisher Behrens test). It is interesting that the membrane potentials ($p<0.001$: two sample t test) and input resistances ($p<0.01$: two sample t test) noted in Ca2K25 were higher than those in Ca2K5 (i.e. calcium concentration is the same in both but the potassium concentration is five fold greater in the former).

The membrane potentials and input resistances recorded in Ca2K25 (choline) were comparable to those in Ca2K25, but those in Ca2K25 (lithium) were significantly lower than both the former populations.

It was interesting to examine the difference in the membrane potential and input resistance before and after fertilization. These values were obtained by subtraction of the respective initial value from the final value. The results are shown in Table 5.2. A positive difference in the membrane potential indicates a depolarization after fertilization and a negative difference indicates a hyperpolarization. Similarily a negative difference in the input resistance indicates a decrease in resistance after fertilization and a positive difference indicates an increase in resistance after fertilization. The results so obtained from eggs bathed in Ca4K5 and Ca2K25 are probably the most conclusive, because of the larger number of results available for analysis. The results obtained in these two types of solutions indicate a depolarization and a decrease in resistance following fertilization. Of the remaining eggs in other solutions, 30 were analysed in

Table 5.3

Summary of the numbers of intracellular recording experiments in which fertilization was attempted and also the numbers of those which were successfully fertilized. The species of sperm used are also noted. All but six fertilizations attempted were homologous fertilizations of zona-free hamster eggs.

SOLUTION	Number of succesful fertilizations / Number of intracellular recordings	SPECIES OF SPERM
Ca2K5	19/27	Hamster
Ca4K5	33/40	Hamster
Ca12K5	1/1	Hamster
Ca42K5	8/9	Hamster
Ca57K5	2/4	Hamster
Ca2K5	1/2	Mouse
Ca4K5	4/4	Mouse
Ca2K25	31/43	Hamster
Ca4K25	4/4	Hamster
Ca7K25	1/1	Hamster
Ca10K25	2/5	Hamster
Ca7K40	1/1	Hamster
Ca2K25 (choline)	4/4	Hamster
Ca2K25 (lithium)	1/1	Hamster

TABLE 5.3

the above manner for changes in the membrane potential. Of these 30 eggs,15 showed a depolarization, 12 showed a hyperpolarization and three showed no change in potential after fertilization. Similarly in three other eggs a decrease in resistance was noted after fertilization.

5.9. Numbers of successful fertilizations performed in the various solutions (Table 5.3)

Table 5.3 shows the number of experiments performed in the various solutions, the numbers which were successful and the species of sperm used.

Most inseminations were carried out in eggs bathed in Ca2K5, Ca4K5 and Ca2K25. It was possible to fertilize zona-free hamster eggs in solutions containing elevated calcium (as high as 57mM) and in solutions containing high potassium. One attempt at fertilization in very high potassium solution (40mM) was successful. It was also possible to fertilize zona-free hamster eggs in solutions in which the sodium chloride was substituted by either choline chloride or lithium chloride.

The reasons for failures of experiments could be one or more of the following in any given experiment:- a) electrical recording became extremely unstable or unreliable - sometimes because of excessive numbers of very motile sperm attached to the egg; b) sperm were not capacitated, they therefore attached to the egg but

did not fuse with it; c) a rapid decrease in the seal between the egg membrane and the intracellular electrode (see Chapter 4), hence it was not possible to maintain a high current clamp potential; d) input resistance of the egg was too low and therefore very large amounts of current were required to current clamp the egg at high potentials. In such eggs the potential would have dropped rapidly had a current clamp been attempted and e) occasionally the act of adding sperm to the recording chamber dislodged the electrode from the egg.

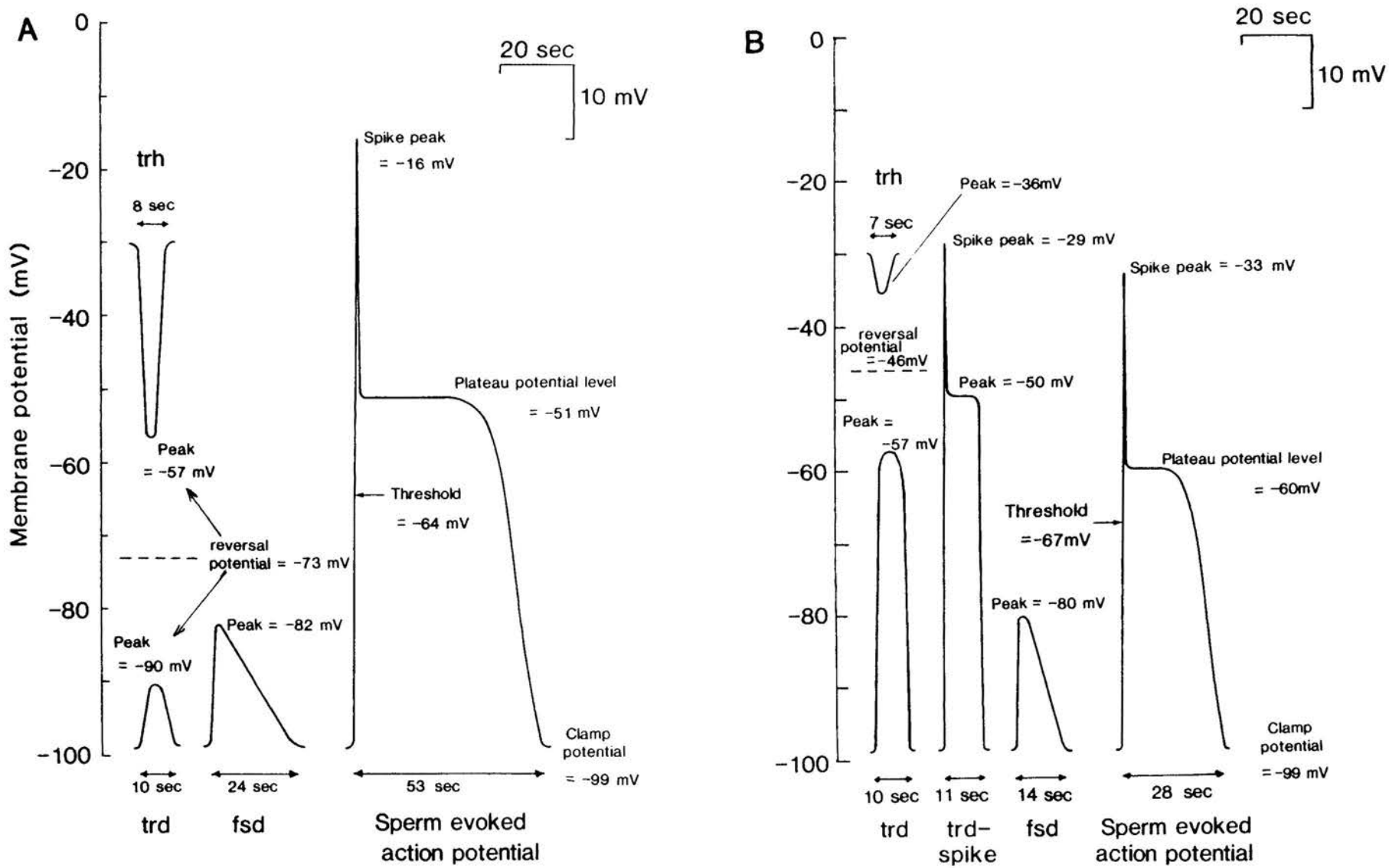
5.10 Different types of electrical responses observed during fertilization of zona-free hamster eggs bathed in normal and high potassium solutions

In Section A and Section B of this chapter experiments described, showed the many types of responses possible during the fertilization of zona-free hamster eggs bathed in normal potassium solutions (Section A) and in high potassium solutions (Section B). These responses have been illustrated in Fig.5.17 (A shows responses in normal potassium solutions, i.e. K = 5mM and B shows responses in high potassium solutions, i.e. K=25mM).

The numerical values for the various measurements of these responses illustrated in Fig.5.17 are the mean values obtained during the course of this study. The mean values were those of two different solutions, i.e. Ca4K5 (normal potassium solution shown in A) and Ca2K25

Figure 5.17

This shows schematically the different types of responses observed during fertilization of zona-free hamster eggs. A are the responses observed in Ca4K5 and B those in Ca2K25. The numerical values shown are the means obtained in this study. All the responses are drawn to the same scale, and can therefore be compared. Also shown are the mean reversal potentials in the two solutions of trhs and trds. All responses illustrated at high potentials have been shown to "start" at a current clamp potential of -99mV (this was the mean current clamp potential during this study: -99 ± 12, n=124).


A
0
-20
-40
-60
-80
-100
Membrane potential (mV)
20 sec
10 mV
trh
8 sec
Peak
= -57 mV
reversal
potential = -73 mV
Peak
= -90 mV
Peak = -82 mV
Spike peak
= -16 mV
Threshold
= -64 mV
Plateau potential level
= -51 mV
Clamp
potential
= -99 mV
10 sec
24 sec
53 sec
trd
fsd
Sperm evoked
action potential
B
0
-20
-40
-60
-80
-100
20 sec
10 mV
trh
7 sec
Peak = -36mV
Spike peak = -29 mV
Spike peak = -33 mV
reversal
potential
= -46mV
Peak = -50 mV
Peak =
-57 mV
Plateau potential level
= -60mV
Threshold
= -67mV
Peak = -80 mV
Clamp
potential
= -99 mV
10 sec
11 sec
14 sec
28 sec
trd
trd-
spike
fsd
Sperm evoked
action potential

(high potassium solution shown in B).

Four types of responses were observed in normal potassium solutions:

a) trh - transient recurring hyperpolarizations: the peak of such a response has been defined as the most hyperpolarized potential reached during the course of such a response

b) trd - transient recurring depolarizations: the peak of such a response has been defined as the most depolarized potential reached during the course of such a response

c) fsd - fast sub-threshold depolarizations: the peak of such a response is also the most depolarized potential reached during its course

d) sperm evoked action potentials: the most depolarized potential of the spike of such an action potential has been called the spike peak. The plateau potential level is the most depolarized level other than the spike itself.

Similar responses to the above mentioned four were also observed in high potassium solutions. But in addition responses which have been called trd spikes were also observed (Fig.5.17B). The spike peak of a trd spike is the most depolarized potential reached by the spike of such a response. The peak of a trd spike is the most depolarized potential other than the spike itself.

The duration of all responses was the time taken

for the potential to return to its initial current clamp or membrane potential.

The five different types of responses observed at fertilization have been classified into two groups. One group is called slow responses and includes trhs, trds and trd spikes. The reason for calling these responses, slow responses, is that their rate of change of potential is slower than the other two types which have been called fast responses (i.e. fsds and sperm evoked action potentials).

The reversal potentials indicated in Figure 5.17A and B were the mean of the reversal potentials of the trhs and trds in normal potassium (Fig.5.17A) and in high potassium (Fig.5.17B) solutions.

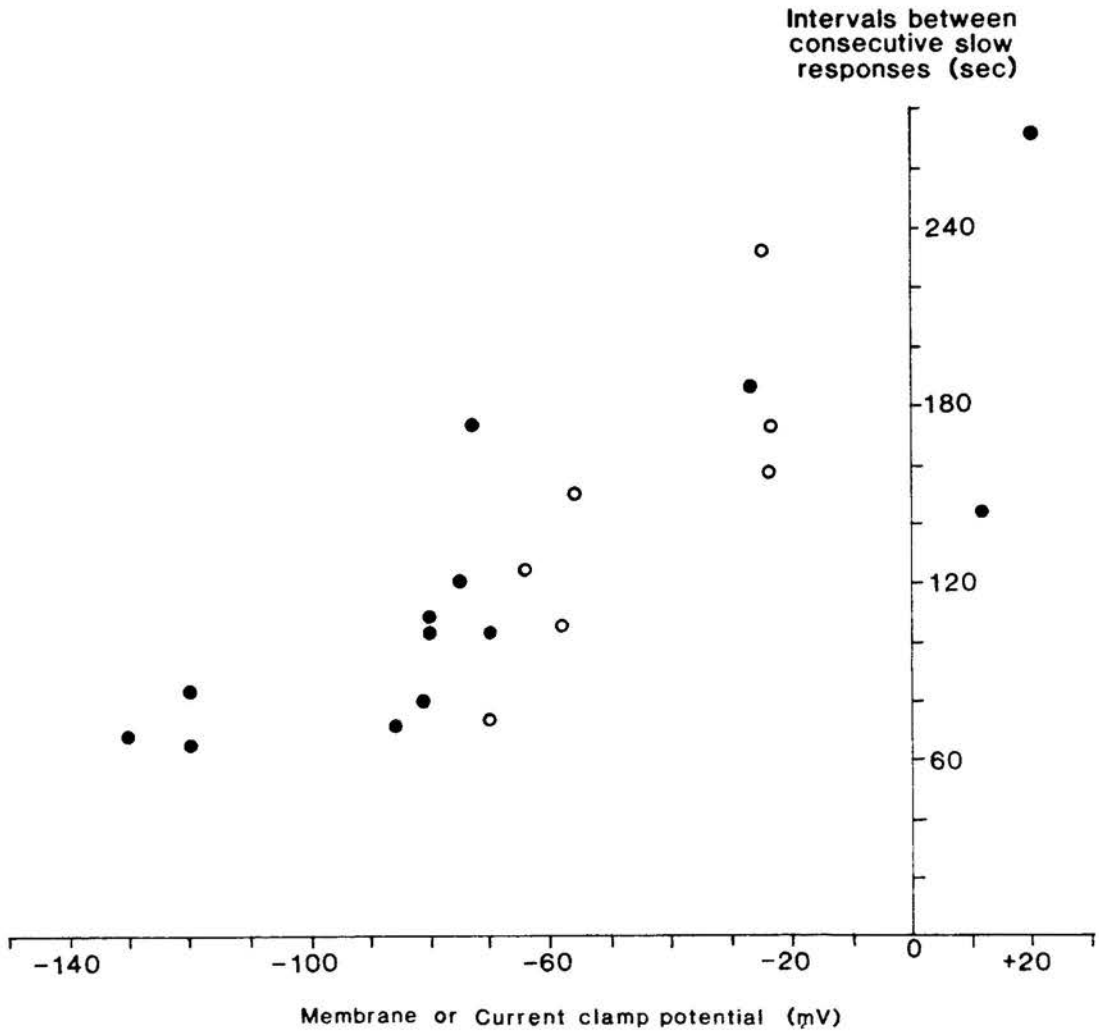
5.11 Frequency of trhs or trds observed at different membrane potentials

If a linear regression analysis is performed on the interval between trhs or trds observed at different membrane potentials, taking into account the whole population of eggs in a given solution, the correlation was very poor. The correlation coefficients of such analyses in Ca2K5, Ca4K5, Ca42K5 and Ca57K5 were 0.23, 0.19, -0.41 and 0.37 respectively .

But if intervals are plotted against membrane potentials for individual eggs then much better correlations are observed. Two such examples are shown in Fig.5.18. The results from one egg bathed in Ca2K5 are indicated by open circles (correlation coefficient

Figure 5.18

The relationship between membrane potential and the interval between consecutive slow responses. Results are for two eggs, one of which was bathed in Ca2K5 (open circles) and the second bathed in Ca2K25 (filled circles).


Intervals between consecutive slow responses (sec)
240
180
120
60
-140
-100
-60
-20
0
+20
Membrane or Current clamp potential (mV)

for which was 0.82, n=7, $p<0.05$). Results from another egg bathed in Ca2K25 are indicated by filled circles in Fig.5.18. The correlation coefficient for these was 0.82, n=13, $p<0.001$. In both eggs the intervals increased as the membrane potential became more depolarized, i.e. the frequency of the slow responses decreases with depolarization.

One might not expect any correlation between frequency and membrane potential for a whole group of eggs, since the frequency might also be related to the number of sperm which have fused with any given egg.

Oscilloscope pictures illustrating this were shown in Fig.5.6.

5.12 Durations of various types of responses observed during fertilization of zona-free hamster eggs with hamster sperm (Table 5.4)

Increasing the calcium in the bathing solution does not affect the duration of trhs in normal potassium solutions (Table 5.4). This is also exemplified by the durations of trds observed in high potassium solutions containing different concentrations of calcium. The durations of trhs in Ca2K25 were not significantly different to those in Ca2K5. Similarly the durations of trds in Ca4K5 were not significantly different to those in Ca4K25. Therefore increasing the potassium concentration with a constant calcium concentration in the bathing medium also, did not

Table 5.4

Summary of the durations of the five types of responses observed during homologous fertilizations of zona-free hamster eggs. Whenever possible the results have been presented in the form mean ± SD (n=), but if the numbers of results were too few, then the actual values have been listed (in italics).

SOLUTION	DURATIONS (seconds) mean ± SD (n=)				
	SLOW RESPONSES			FAST RESPONSES	
	trh	trd	trd spike	fsd	sperm evoked action potential
Ca2K5	6±3 (23)	26 208		18±11 (7)	
Ca4K5	8±2 (31)	10±5 (17)		24±25 (36)	53 ±74 (8)
Ca12K5				15	
Ca42K5	9±3 (33)	10			
Ca57K5	8±2 (9)	7 7 7		7	
Ca2K25	7±1 (8)	10±4 (50)	11±3 (23)	14±12 (25)	59±8 (5)
Ca4K25	9	10±3 (8)	9	5 6 8 11	
Ca7K25			9 9 14 16		
Ca10K25		13±2 (10)	10	10 35	12
Ca7K40				42	
Ca2K25 (choline)		12±4 (18)	13 15 16 17	17 32 57 67	
Ca2K25 (lithium)		8 14		48	

TABLE 5.4

affect the durations of slow responses.

The durations of trds were larger than those of trhs in Ca4K5 ($p<0.05$: Fisher Behrens test) and in Ca2K25 ($p<0.01$: Fisher Behrens test). The durations of trd spikes were not larger than the durations of trds in Ca2K25 ($p>0.05$: Fisher Behrens test).

Eighteen trds were observed on fertilization of eggs bathed in Ca2K25 (choline). The duration of these was similar to those in Ca2K25.

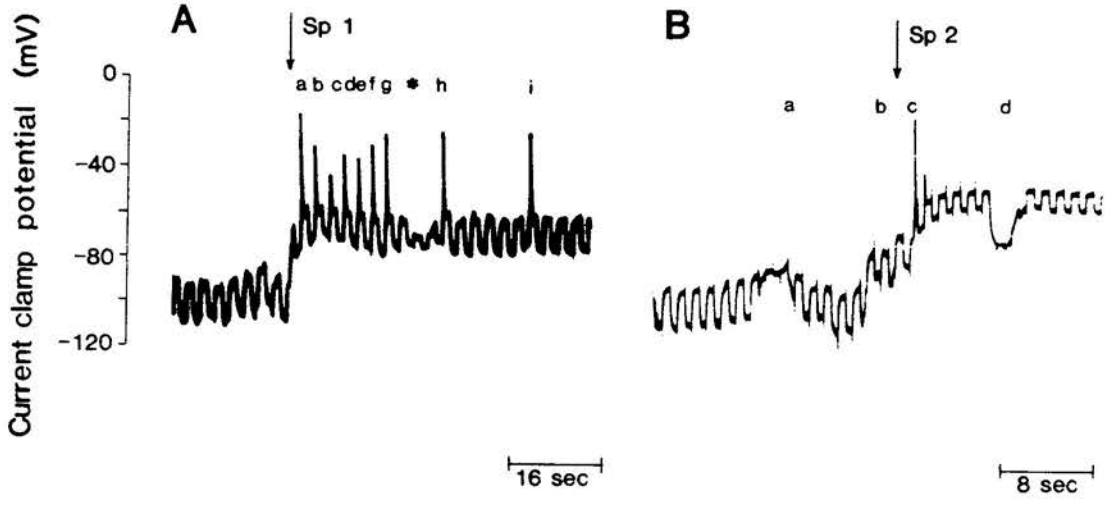
Durations of fsds in Ca2K5 and Ca4K5 were significantly larger than any type of slow response observed (trhs and trds are the only slow responses observed in normal potassium solutions). It is evident from the standard deviations that the durations of the fsds are more variable than the slow responses. Similarly the durations of fsds in Ca2K25 were larger than the durations of any of the three types of slow responses oberved in this solution.

The durations of the sperm evoked action potentials were even larger than the fsds observed in the same solution (e.g in Ca4K5 or Ca2K25).

Occasionally when an fsd or a sperm evoked action potential was recorded the membrane potential did not return to its value prior to the response. Such "permanent plateaux" have been observed twice in Ca2K5 (one of which was an fsd and the other a sperm evoked action potential), nine times in Ca4K5 (four of which were fsds and the remainder were sperm evoked action potentials) and three times in Ca2K25 (two of which

Figure 5.19

A. Fsd caused by the first sperm fusion in a zona-free hamster egg bathed in Ca4K5. This had a permanent plateau. The egg was current clamped at -92mV prior to insemination and hyperpolarizing current pulses passed through the electrode to monitor the conductance. The arrow indicates the time of the first sperm fusion. Letters a-i refer to individual anode break spikes caused by hyperpolarizing pulses (0.4Hz, 1.2sec, 0.09nA). The asterisk denotes a trh response. B. A sperm evoked action potential caused by the second fusion (arrow), recorded in an egg bathed in Ca2K5. Letters a-d refer to electrical events which have been described in the text. The egg was current clamped at -86mV prior to insemination and the hyperpolarizing pulses were 0.4Hz, 1.2sec, 0.09nA.


A
Sp 1
a b c d e f g * h i
0
-40
-80
-120
Current clamp potential (mV)
16 sec
B
Sp 2
a b c d
8 sec

were fsds and the third was a sperm evoked action potential). Four of these have been illustrated in Figures 5.3, 5.5, 5.12 and 5.13.

Figure 5.19A shows an oscilloscope picture of an fsd recorded at the instant of hamster sperm fusion with a zona-free hamster egg. This egg was current clamped at -92mV, prior to insemination. Hyperpolarizing pulses were passed continuously to monitor conductance changes. The fsd caused by sperm fusion plateaued at about -64mV. In a further nine minutes of recording 9 trhs were recorded on this plateau (the plateau gradually depolarized to -53mV, whilst still under current clamp). The first of these trhs is shown in the oscilloscope picture (Fig.5.19A), occurring 18 seconds after the sperm fusion and is marked by an asterisk. The reversal potentials of the first eight trhs were -75, -74, -72, -72, -72, -66, -72 and -75mV respectively (i.e. the reversal potential of the trh shown in Fig.5.19A was -75mV). Since conductance was being continuously monitored throughout this experiment it was possible to calculate the reversal potential of this fsd, which was found to be +132mV. Another feature noted from this oscilloscope picture and also observed in other experiments, is that the input resistance of the egg is lower during the plateau as indicated by the size of the electrotonic potentials recorded. At the beginning of this fsd anode break responses were observed (due to the

hyperpolarizing current pulses being used to monitor the conductance) as marked by letters. The spike peak of the anode break responses may be indicative of the cytosolic calcium concentration. As the anode break spike peak hyperpolarizes, then theoretically the cytosolic calcium must be increasing. If this assumption is correct, then after the fsd, the cytosolic calcium rises to a peak at about 8 seconds (minimal anode break spike peak height occurred at spike label "c") and then starts to decrease (anode break spike peak height reaches a maximum at spike "g"). Spike "g" was then followed by a trh. Two further anode break spikes were observed ("h" and "i").

The above example was one in which the first sperm fusion elicited an fsd with a plateau. The example shown in 5.19B is of the second sperm fusion in an egg bathed in Ca2K5, which elicited a sperm evoked action potential with a permanent plateau. The egg was current clamped at -86mV, prior to insemination. The first sperm fusion in this egg elicited an fsd, which was followed by three trds. The third of these is shown in Fig.5.19B and is marked "a". About four seconds after this trd was a depolarization ("b") which was immediately followed by the action potential ("c"). This action potential had a plateau which had not returned to the initial current clamp potential by the end of the experiment (33.4 minutes after the action potential). After the action potential 18 trhs were noted, the first of which is shown in Fig.5.19B ("d").

There are many interesting features about this record. Firstly the resistance during the plateau is lower than that prior to the action potential (about 47% of its initial value). The reversal potential of the trd ("a") was -70mV, whereas of the trh ("d") it was -62mV. It might be that the more positive reversal potential of the slow response superimposed on the plateau is linked to the lower resistance during the plateau, i.e. during the plateau the potassium conductance is greater.

During the depolarization marked "b", there was a small drop in resistance. Such a depolarization has previously been called a predepolarization. Superimposed on this predepolarization was the action potential (which caused a further, bigger increase in the conductance). This raises the possibility that the sperm fusion causes the predepolarization, which then triggers a further event or events leading to a further drop in the input resistance of the egg and also an action potential. But there may be situations when this secondary event is not triggered and the predepolarization is seen electrically as an fsd.

5.13 Potentials at the peaks of the slow responses obtained during fertilization and their reversal potentials (Table 5.5)

The peak potentials of the trds observed in Ca4K5 were -90 $\pm$ 12mV, n=18 and the peak potentials of trhs

in the same solution were -57±21mV, n=29. Therefore it appears that "on average" no trhs or trds would be observed if the membrane potential (or current clamp potential) lies between -57mV and -90mV (for an egg bathed in Ca4K5). These limits are the mean peak potentials of trhs and trds in Ca4K5. This "band of no response" may be wider, and is probably dependent on the input resistance of the egg.

It is interesting that the peak of the trd spikes in Ca2K25 was on average more depolarized by 8mV, than the peaks of the trds in the same solution. The means of the two populations were significantly different ($p<0.01$: Fisher Behrens test). It is possible that the "extra" increase in cytosolic calcium caused by the calcium influx from the bathing medium during the spike maybe responsible for this more depolarized peak. Since a greater increase in cytosolic calcium would theoretically take the peak for any of the three slow responses closer to the potassium reversal potential, within limits. A similar sort of logic may be used to explain the shift in the peak potential towards the potassium reversal potentials, with increasing external calcium (both in normal and high potassium solutions). For example in high potassium solution, let's assume that the reversal potential is about -40mV. The mean peaks of the trds were -57mV, -50mV and -43mV in Ca2K25, Ca4K25 and Ca10K25 respectively (the two populations of trds observed in Ca2K25 and Ca10K25 were significantly different: $p<0.01$: Fisher Behrens test),

Table 5.5

Summary of the peak potentials and the reversal potentials of the three types of slow responses observed during homologous fertilization of zona-free hamster eggs. Results are in the form mean ± SD (n=) or the actual values are quoted (in italics).

SOLUTION	PEAK POTENTIALS (mV)			REVERSAL POTENTIAL (mV)		
	trh	trd	trd spike	trh	trd	trd spike
Ca2K5	-35±13 (21)	-86 -88		-69±9 (40)	-72±13 (10)	
Ca4K5	-57±21 (29)	-90±12 (18)		-77±7 (18)	-69±8 (11)	
Ca12K5				-75 -86		
Ca42K5	-46±11 (33)	-69		-69±5 (8)		
Ca57K5	-39±9 (9)	-86 -89 -91 -97				
Ca2K25	-36±3 (8)	-57±18 (44)	-49±5 (23)	-51±9 (15)	-41±7 (8)	-42
Ca4K25	-39	-50±6 (8)	-58		-35 -41 -47	
Ca7K25			-36 -42 -42 -46	-31	-30	-35 -41
Ca10K25	-25	-43±10 (10)	-50 -50	-37±3 (5)	-40 -41 -42	
Ca2K25 (choline)		-61±13 (18)	-46 -48 -49 -50	-42	-32	
Ca2K25 (lithium)		-76 -93		-37 -43		

TABLE 5.5

i.e. as the calcium concentration in the bathing medium was increased, the peak potential became closer to the potassium reversal potential (-43mV in Ca10K25 compared to -57 in Ca2K25). Similarly a hyperpolarizing shift in the mean peak potential of trhs was noted, as the calcium concentration was increased, in normal potassium solutions. For example the peaks of trhs in Ca2K5 and Ca4K5 were -35 $\pm$ 13mV, n=21 and -57 $\pm$ 21mV, n=29 respectively ($p<0.01$: Fisher Behrens test). In this reasoning the results obtained in very high calcium (Ca42K5 and Ca57K5) solutions were ignored, because of the large deviation from the norm.

The peak of the trd was not significantly different in Ca2K25 (choline) to that in Ca2K25. Similarly, there was no significant difference in the peak potential of trhs in Ca2K5 and Ca2K25.

The reversal potential is a much better parameter to measure, since unlike the peak potential it takes into account the membrane potential and the input resistance of any given egg. Theoretically the reversal potential obtained for trhs and trds should be the same in any given solution. They were found to be in close agreement in Ca2K5 (Table 5.5).

According to the Nernst equation, one would expect for a five fold change in the external potassium concentration a shift in the reversal potential of 42mV, i.e. $60 \log_{10} 5$ (all other ions being constant). Assuming that the reversal potential in Ca2K5 is equal

to -70.5mV (average of the mean reversal potential of trhs and trds in Ca2K5) and that in Ca2K25, it is -46mV (average of the mean reversal potential of trhs and trds in Ca2K25 - see Table 5.5) then the shift is calculated to be 24.5mV (i.e. 70.5-46). This would indicate that the conductance change observed during the course of a trh or a trd is not solely due to a potassium conductance.

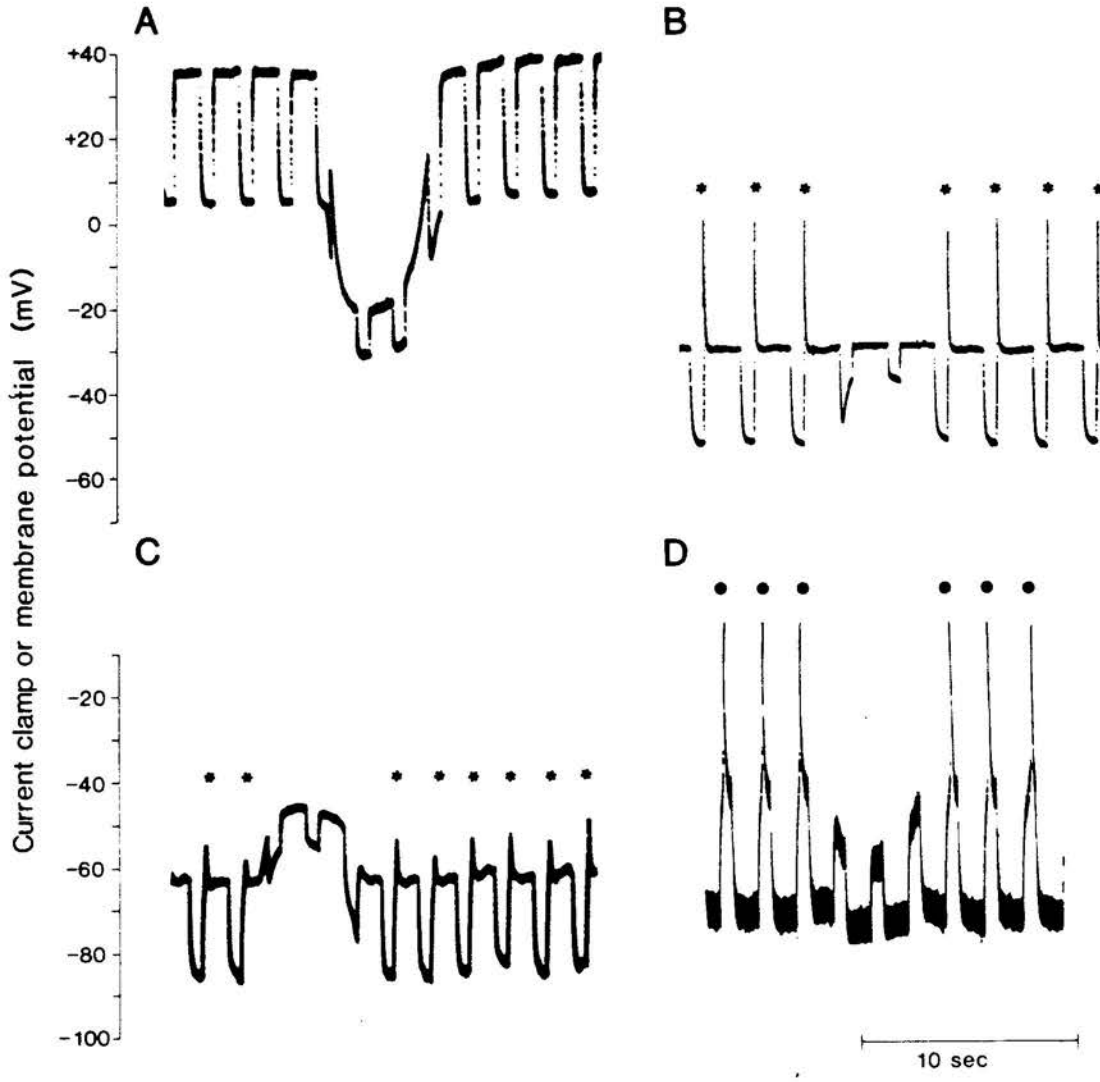
There was no significant difference between the reversal potential of trhs in Ca2K5 and Ca4K5 ($p>0.50$: two sample t test). Similarly there was no significant difference in the reversal potentials of trds in Ca2K5 and Ca4K5.

But the reversal potentials of trhs in Ca2K25 and Ca10K25 were significantly different, ($p<0.01$: Fisher Behrens test). As mentioned above, the peak potential in Ca10K25 was also more depolarized than that in Ca2K25. If indeed the reversal potentials measured in Ca4K5 and Ca10K25 are better estimates of the reversal potentials in these two types of potassium solutions, then there is a shift of 40mV (i.e. reversal potential of trh in Ca4K5 minus the reversal potential of trh in Ca10K25 = 77-37) in the reversal potential in going from the normal potassium solution to the high potassium solution. This is in closer agreement with that expected from the Nernst equation.

The closer approximation of the reversal potential of these trh responses to the potassium equilibrium potential, in bathing solutions containing higher

Figure 5.20

This shows four oscilloscope pictures of responses observed during fertilization of four different zona -free hamster eggs with hamster sperm. In all these examples the reversal potential was calculated by monitoring the conductance. This was done by passing either hyperpolarizing current pulses (as in A,B and C) or depolarizing pulses (as in D) through the electrode. Depending on the membrane potential or current clamp potential of the egg, such pulses could elicit anode break responses (as in B and C, marked by asterisks) or electrically evoked action potentials (as in D, marked by filled circles). The latter arising because depolarizing pulses were being passed through the electrode. A is a trh recorded in an egg fertilized in Ca2K25 (reversal potential equals -54mV, current clamp potential equals +37mV). B is a trd recorded in an egg fertilized in Ca7K25 (reversal potential equals -30mV, membrane potential equals -32mV). C is a trd recorded in an egg fertilized in Ca2K25 (choline) (reversal potential equals -32mV, current clamp potential equals -62mV). D is a trh recorded in an egg fertilized in Ca42 Na80 (reversal potential equals -82mV, current clamp potential equals -72mV). A-C were in high potassium and D was in normal potassium. The pulses were all 0.5Hz, 0.6 seconds (in A-D) but the magnitude of each pulse was 0.3nA in A, 0.1nA in B, 0.2nA in C and 0.2nA (depolarizing) in D.


A
B
C
D
+40
+20
0
-20
-40
-60
-20
-40
-60
-80
-100
Current clamp or membrane potential (mV)
10 sec

concentrations of calcium, may also be due to the higher input resistances of eggs bathed in these solutions (see 5.26).

It would be nicer to measure the reversal potential for these slow responses in the same egg, whilst bathed in solutions containing different concentrations of potassium. Unfortunately, such experiments were not performed in this study.

Figure 5.20 illustrates four examples of slow responses, recorded in four eggs bathed in different solutions.

5.14 Spike peaks of trd spikes, electrically evoked action potentials and sperm evoked action potentials (Table 5.6)

The peak potentials of the spikes (spike peak) in trd spikes, electrically evoked action potentials and sperm evoked action potentials (Table 5.6) in eggs bathed in Ca2K25 were found to be -29 ± 10mV, n=22; -22 ± 10mV, n=40 and -33 ± 13, n=11 respectively. The spike peak of the sperm evoked action potential was more hyperpolarized than that of the electrically evoked action potential in Ca2K25 ($p<0.02$: two sample t test).

In ten eggs bathed in Ca2K25 a measure of the peaks of both sperm and electrically evoked action potentials was obtained. The peak of the sperm evoked action potential was more hyperpolarized than the peak

Table 5.6

A summary of the spike peaks of trd spikes, electrically evoked action potentials and sperm evoked action potentials in various solutions. (No trd spikes were observed in normal potassium solutions). The peak potentials and amplitudes of fsds are also listed. The column on the extreme right shows the reversal potentials of fsds. Results are expressed as mean ± SD (n=) or the actual values are quoted (in italics).
All results were from homologous fertilizations of zona -free hamster eggs.

SOLUTION	SPIKE PEAK (mV)			Peak of fsd (mV)	Amplitude of fsd (mV)	Reversal potential of fsd (mV)
	trd spike	Electrically evoked action potential	Sperm evoked action potential			
Ca2K5		-22±10 (9)	-4 -7	-83±15 (8)	18±12 (8)	+39±53 (15)
Ca4K5		-9±14 (27)	-16±9 (12)	-82±18 (39)	19±9 (40)	+53±55 (14)
Cal2K5		-4		-86	10	+78
Ca57K5				-95	11	
Ca2K25	-29±10 (22)	-22±10 (40)	-33±13 (11)	-80±12 (27)	16±11 (27)	-34
Ca4K25	-31	+4 -12 -26 -33		-68 -86 -96 -100	8 12 23 39	
Ca7K25	+7 +5 +4 +4	+16		-66		
Cal0K25	-12 -38	0 0 0		-64 -73	11 12	
Ca20K25		-30				
Ca7K40		-32				
Ca2K25 (choline)	-36 -49 -52 -58	-35±6 (5)		-64 -70 -76 -78	10 11 20 32	
Ca2K25 (lithium)				-84 -115	11 22	

TABLE 5.6

of the electrically evoked action potential in these 10 eggs ($p<0.05$, $n=10$; Wilcoxon signed rank test).

A similar comparison of the spike peak of electrical and sperm evoked action potentials recorded in Ca4K5 indicated that these two populations were not significantly different ($p>0.05$; Fisher Behrens test). Although the mean values of these two populations indicated that the spike peak of the sperm evoked was more hyperpolarized than that of the electrically evoked action potential. In six eggs bathed in Ca4K5 a measure of the peaks of both sperm and electrically evoked action potentials was obtained. No significant difference was found between each pair of values, for each egg ($p>0.05$, $n=6$; Wilcoxon signed rank test).

The spike peak of the electrically evoked action potential became more depolarized on increasing the calcium concentration in the bathing medium (for a given potassium concentration). For example that in Ca4K5 was -9 ± 14mV, $n=27$ and in Ca2K5 was -22 ± 10mV, $n=9$ (the two populations being significantly different $p<0.01$; two sample t test). From the Nernst equation, one would expect a doubling of the divalent ion concentration to produce a shift of 9mV, i.e. $30\log_{10}2$. The above results indicate a mean shift of 13mV (i.e. 22-9).

Only five electrically evoked action potentials were recorded in Ca2K25 (choline). The mean $\pm$ SD was found to be -35 ± 6mV, $n=5$, which was significantly different to that in Ca2K25 ($p<0.01$; Fisher Behrens

test). This indicates that the spike peak of the electrically evoked action potential in Ca2K25 (choline) was more hyperpolarized than that in Ca2K25. Therefore, either there is a sodium contribution to the spike (in the electrically evoked action potential), or choline blocks the calcium channels.

The spike peak of the electrically evoked action potential was not significantly different in Ca2K5 and Ca2K25 (-22±10mV, n=9 and -22±10mV, n=40 respectively).

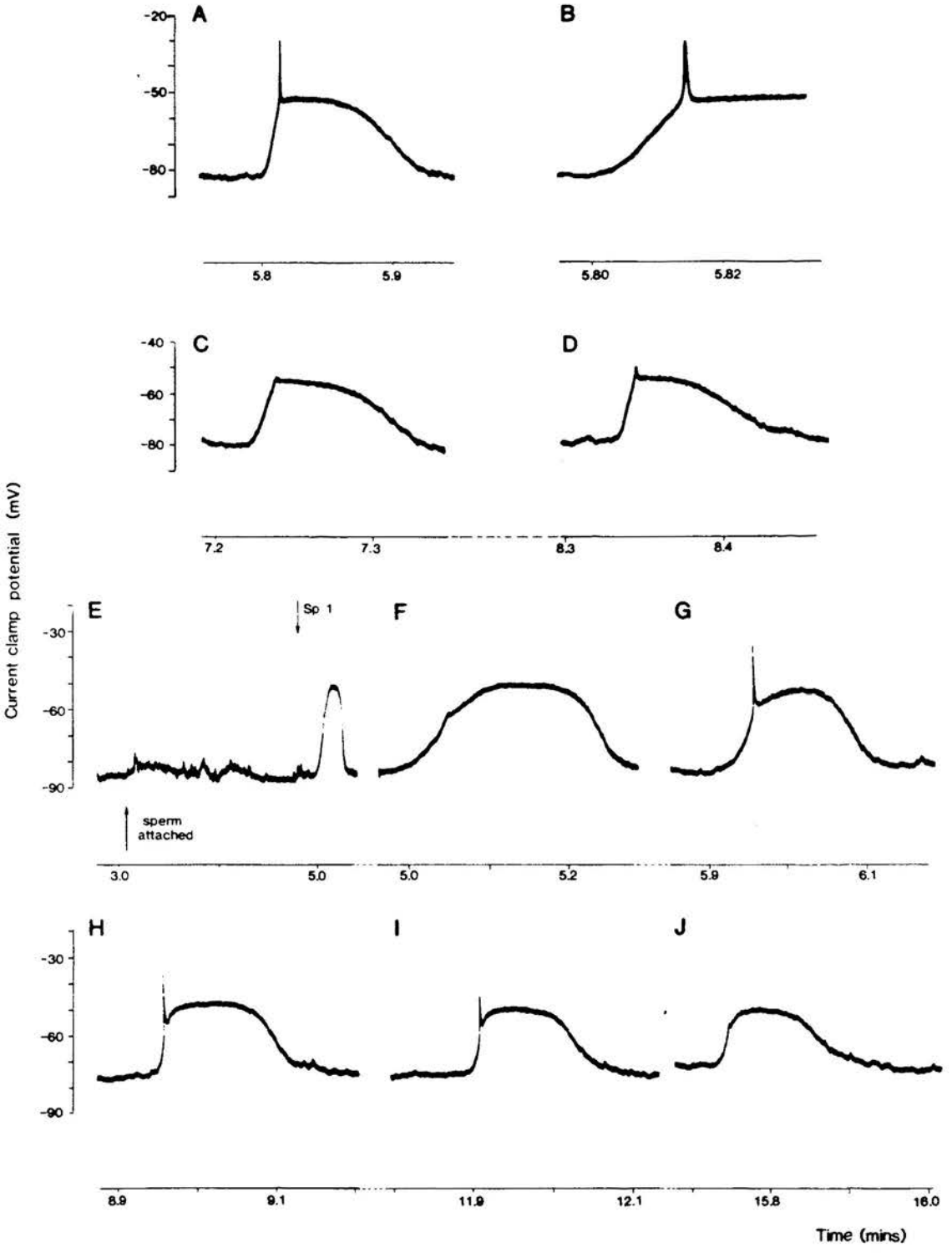
5.15 Spike peaks of consecutive trd spikes

Figure 5.21A,C and D show three consecutive trd spikes recorded in a zona-free hamster egg fertilized by hamster sperm in Ca2K25. Although the current clamp potential and amplitude is almost identical (in order of first to last the current clamp potential was -82mV, -78mV and -80mV and the amplitudes in order were 28mV, 27mV and 26mV) the spike peaks were very different. Indeed, in the second of these three it was almost negligible. This fluctuation of the spike peak could be an indication of the altering cytosolic calcium (the spike peaks were -30mV, -53mV and -49mV, in order). None of these three was associated with a sperm fusion. B is a faster sweep speed oscilloscope picture of the rising phase of the trd spike shown in A.

Figure 5.21E-J illustrates this point also, in another egg fertilized in Ca2K25. About 3.1 minutes after insemination of the recording chamber the sperm

Figure 5.21

Oscilloscope pictures of responses observed during homologous fertilization of zona-free hamster eggs, bathed in Ca2K25. A,C and D are the three consecutive responses recorded in an egg current clamped to a potential of -80mV. B is an oscilloscope picture of the rising phase of the first trd spike shown in A, at a faster sweep speed. E-J are the responses recorded in another current clamped egg. E shows the incident of sperm attachment (arrow below the picture) and the approximate instant of sperm fusion (arrow above the picture) in relation to the "slow response" shown at the end of E and also in F. G-J are the four responses which followed. Pictures F-J are all at the same sweep speed.


A
B
C
D
E
F
G
H
I
J
-20
-50
-80
-40
-60
-30
-90
Sp 1
sperm attached
5.8
5.9
5.80
5.82
7.2
7.3
8.3
8.4
3.0
5.0
5.0
5.2
5.9
6.1
8.9
9.1
11.9
12.1
15.8
16.0
Current clamp potential (mV)
Time (mins)

attached to the egg (shown by the arrow below the oscilloscope picture in Fig.5.21E). At 4.8 minutes a sperm fused with the egg (i.e. became straight and immotile, Fig.5.21E). This was followed 14 seconds later by a trd, shown in both Fig.5.21E and at a faster sweep speed in Fig.5.21F. The responses which followed the sperm fusion are shown in Fig.5.21F-J. The peaks (not the spike peaks) of these five responses were -50, -54, -49, -51 and -50mV respectively. Similarly the durations were 16, 12, 10, 9 and 11 seconds in order. These five responses were superimposed on a depolarization (the current clamp potential prior to the first trd in Fig.5.21F was -86mV and that prior to the fifth shown in Fig.5.21J was -72mV. This depolarizing shift could therefore be an explanation of the reduction in the spike peak (i.e. hyperpolarization of the spike peak) in the responses shown in Fig.5.21 G-J. Although another possibility could be an increase in cytosolic calcium. But the first slow response (Fig.5.21F) was longer in duration than the rest, it was current clamped at the most hyperpolarized potential of all five slow responses and its peak was actually more depolarized than that of the second (shown in Fig.5.21G). This indicates that the sperm fusion increased the cytosolic calcium above the levels preceding the subsequent responses. This may be an explanation for both the longer duration and the negligible spike in Fig.5.21F.

5.16 Peak potentials and amplitudes of fsds (Table 5.6)

The peak potentials of fsds were found to be in close agreement in the three different solutions, i.e. -83±15mV, n=8 in Ca2K5; -82±18mV, n=39 in Ca4K5 and -80±12mV, n=27 in Ca2K25.

The amplitude of fsds were similar also: 18±12mV, n=8 in Ca2K5, 19±9mV, n=40 in Ca4K5 and 16±11, n=27 in Ca2K25. The range of amplitudes was 6mV to 40mV in Ca2K5 and Ca4K5, whereas in Ca2K25 it was 6mV to 42mV.

Since the average current clamp potential in this study was -99±12mV (n=124) then an fsd of amplitude approximately equal to 18mV would be sufficient to depolarize the membrane or current clamp potential to about -81mV. This as we shall see later in this chapter is more hyperpolarized than the threshold for a sperm evoked action potential (in any of the above three solutions) and hence would not cause a sperm evoked action potential.

The largest fsd recorded had an amplitude of 42mV, and the egg (bathed in Ca2K25) in which it was recorded, was current clamped at -114mV. Therefore the peak potential for this particular fsd was -72mV. The threshold for the sperm evoked action potential in eggs bathed in Ca2K25 was -67mV (see 5.22), and thus since this fsd was subthreshold, no sperm evoked action potential was observed.

5.17 Reversal potentials of fsds (Table 5.6)

The reversal potential of fsds was calculated in a manner similar to that of the slow responses, described in 5.2. The possible error in the calculation of such reversal potentials of fsds was discussed in 5.3 with reference to Figure 5.8.

The reversal potentials of fsds were found to be $+39\pm53$mV, n=15 and $+53\pm55$mV, n=14 in Ca2K5 and Ca4K5 respectively. Although the means seemed to indicate that the reversal potential is more depolarized in Ca4K5 than that in Ca2K5, no significant difference was found between the means of the two populations ($p>0.4$; two sample t test).

Occasionally the amplitude of fsds was observed to be very small, indeed the smallest fsd observed in Ca2K5, Ca4K5 and Ca2K25 was 6mV.

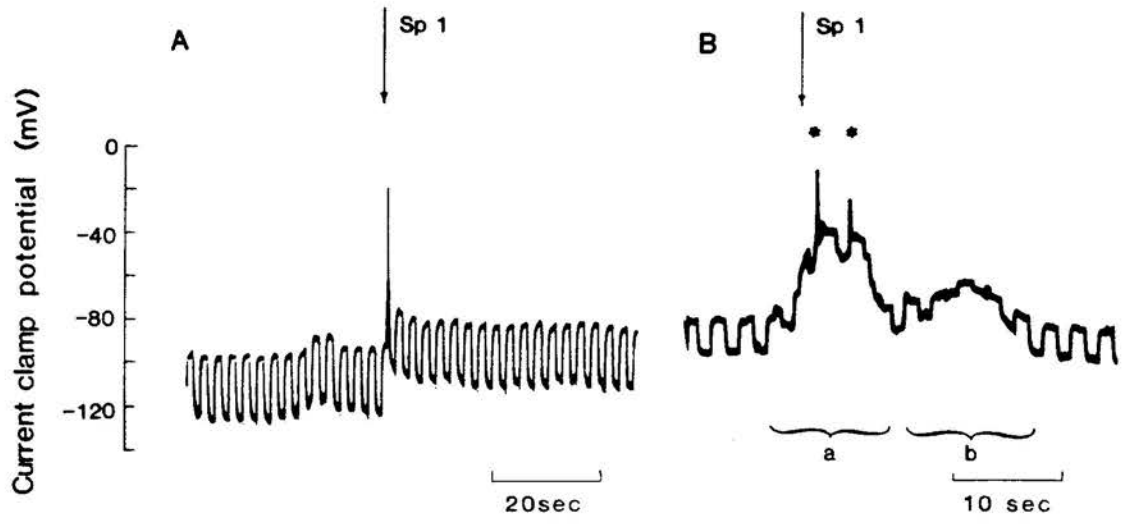
The large range of reversal potentials and the standard deviations about the mean, for these fsds is probably an indication of the error involved in the calculation and also the variability in the response (because of the variable input resistances of different eggs in the same solution). The error is likely to be larger in smaller input resistance eggs.

The ranges of the calculated reversal potentials in Ca2K5 and Ca4K5 were -26mV to +134mV and -23mV to +132mV respectively.

The doubling of the calcium ion concentration in the bathing medium would be expected to produce a shift

Figure 5.22

Responses accompanying the first fusion in two eggs bathed in Ca2K5, when fertilized by hamster sperm. A. the egg was current clamped at -100mV prior to insemination, and the fusion elicited an action potential (indicated by the arrow above the picture). The egg in B was current clamped at -86mV. Fusion in this egg caused a fast response ("a") which was followed shortly by a trd ("b"). The two spikes marked by asterisks, observed during the fast response were anode break spikes caused by the hyperpolarizing pulses being used to monitor the conductance changes. The hyperpolarizing pulses in A were 0.2nA, 0.40Hz, 1sec and in B 0.1nA, 0.40Hz, and 1 sec.


A
Sp 1
B
Sp 1
Current clamp potential (mV)
0
-40
-80
-120
20sec
a
b
10 sec

of 9mV (i.e. 30 $\log_{10}2$) in the equilibrium potential of calcium. If we consider just the mean values of the reversal potential in Ca2K5 and Ca4K5, then doubling of the calcium in this study produced a shift of 14mV (i.e. 53-39), which is in fairly close agreement with Nernst (bearing in mind the possible errors and the fact that statistical analysis indicated that the two populations were not significantly different).

In Figure 5.22A is an example of a sperm evoked action potential recorded in a zona-free hamster egg bathed in Ca2K5. The current clamp potential was -100mV and the reversal potential of the response ignoring, the spike (strictly, it is the reversal potential of the plateau of the sperm evoked action potential) was +28mV. Such calculations of the reversal potential of sperm evoked action potentials were not included in the above data. Figure 5.22B illustrates the difference between the reversal potentials of fast and slow responses. This was made possible, because quite frequently a slow response was observed to follow a fast response (the latter being evoked by a sperm fusion). This egg was also bathed in Ca2K5 and the first sperm fusion with it was accompanied by what appears to be an fsd (labelled "a" in Figure 5.22B). But it is likely that it was in fact a sperm evoked action potential and not an fsd, since its peak was -35mV. This emphasises how the continuous monitoring of conductance, can occasionally "mask" the

true nature of the response. The two spikes marked with asterisks in Figure 5.22B were anode break responses caused by the hyperpolarizing pulses. Almost as soon as this fast response had returned to the initial current clamp potential, a trd was observed (labelled "b" in Figure 5.22B). The reversal potentials of the fast and slow response were +25mV and -85mV respectively (the former was not included in the above mentioned analysis).

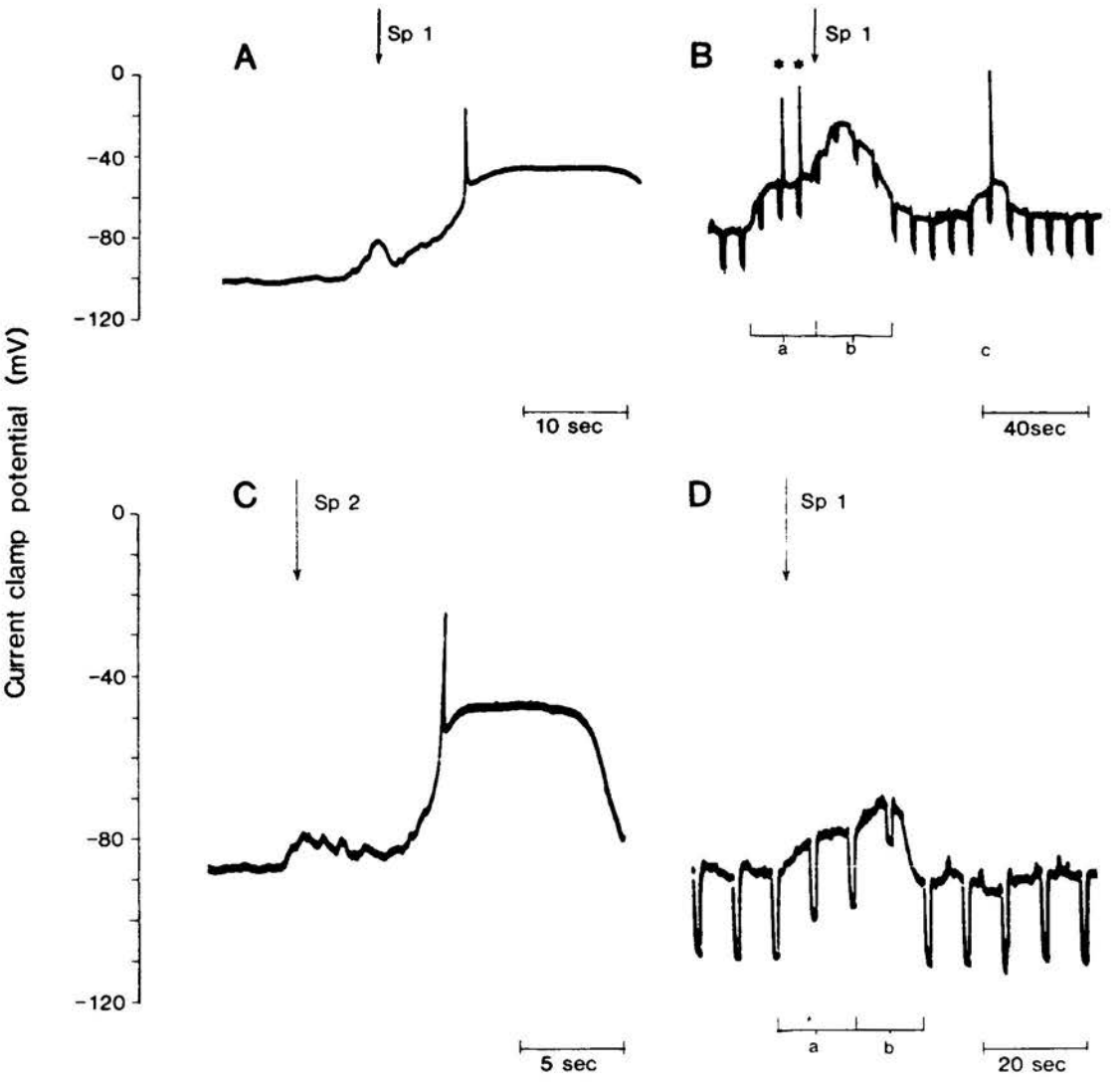
5.18 Predepolarizations prior to trds, trd spikes, fsds and sperm evoked action potentials accompanying sperm fusions

Occasionally, before sperm evoked action potentials depolarizations were observed which were in some cases as large as +30mV. These were called predepolarizations. They were not fsds because they did not have fast rise times. A sperm fusion was associated with either an fsd or a sperm evoked action potential, so could therefore be described as being mutually exclusive. Many examples of sperm evoked action potentials with predepolarizations have already been illustrated (Figures 5.3, 5.4, 5.11C, 5.11D, 5.12A, 5.13A, 5.13Bc and 5.19B).

On occasions a predepolarization was seen to precede trd responses. Examples of these have already been noted (Figures 5.14Bd, and 5.21E). A brief recapitulation of those events in Figures 5.14Bd and 5.21E is given here. In those two cases the sperm

Figure 5.23

Oscilloscope pictures of responses which accompanied sperm fusions (marked by arrows). A was recorded in a zona-free hamster egg (bathed in Ca2K25, current clamped at -102mV), in synchrony with the first sperm fusion. The second sperm fusion in the same egg elicited the response shown in C, 11 minutes later. B was evoked by the first sperm fusion in an egg fertilized in Ca4K5. Asterisks denote anode break responses, and the letters a-c refer to the responses described in the text. Hyperpolarizing pulses were used to monitor the conductance (0.12Hz, 1.2sec, 0.1nA). D - recorded during the first fusion of another egg, also bathed in Ca4K5. Letters "a" and "b" refer to the two phases of the response shown. The hyperpolarizing pulses were 0.15Hz, 1.2sec, 0.1nA.


A
Sp 1
0
-40
-80
-120
10 sec
B
Sp 1
a
b
c
40sec
C
Sp 2
0
-40
-80
-120
5 sec
D
Sp 1
a
b
20 sec
Current clamp potential (mV)

fusion was very quickly followed by a trd. Indeed in Fig.5.14Bd the trd actually appeared to be superimposed on the predepolarization and in Fig.5.21E the trd was longer in duration than average (of the subsequent slow responses observed in that egg). But most important these were not trd spikes even though the subsequent slow responses were. Two examples of predepolarizations preceding trd spikes are shown in Figure 5.23A and C. Both these are recorded in the same egg bathed in Ca2K25. That shown in A was elicited by the first sperm fusion and that in C by the second. The duration of the response shown in A was 16 seconds and that of the response in C was 12 seconds.

Since sperm evoked action potentials were sometimes preceded by predepolarizations, one might therefore also expect some fsds to be preceded by such responses. But due to the similar size of fsds and predepolarizations they would be extremely difficult to distinguish. On four occasions during conductance measurements, it was possible to distinguish the two separate conductance changes, each one was associated with a separate depolarization phase. One such example is shown in Fig.5.23B. In this egg bathed in Ca4K5 hyperpolarizing pulses (0.12Hz, 1.2secs, 0.1nA) were being used to monitor conductance changes. The first depolarizing phase called "a" had a reversal potential of +140mV, superimposed on this was a second depolarizing phase ("b"), associated with sperm fusion,

having a reversal potential of +21mV. The first phase ("a") was associated with a very small conductance increase (about 10 per cent), whereas the second phase ("b") was asociated with a further conductance increase of about 50 per cent. Two anode break responses (marked by asterisks) were observed during "a" elicited by the hyperpolarizing pulses. The reversal potential of "b" is compatible with that of the reversal potentials calculated for fsds. The phase denoted as "a" has been interpreted as being a predepolarizing phase. But "b" cannot be called an fsd, (since its peak potential is about -23mV) because by definition an fsd is subthreshold. The response marked "c" in Fig.5.23B was not associated with another sperm fusion. It was short lasting (about 16 secs) with a reversal potential of -29mV.

In another egg bathed in Ca4K5, sperm fusion caused another "two phase" response shown in Fig.5.23.D. The first phase "a", associated with sperm fusion, had a reversal potential of +17mV, whereas the second phase "b" had a reversal potential of -62mV. This has been interpreted as being a trd preceded by a predepolarization (as discussed above). Had this particular egg been inseminated in Ca2K25, the trd (the second phase) would presumably have been a lot larger. This example illustrates that it is easier to discriminate the two phases in a trd with a predepolarization, when recorded in an egg bathed in high potassium solution than in one bathed in normal

potassium solution.

5.19 Is there any depolarizing event prior to the first trh recorded in an egg fertilized at low membrane potentials?

The electrical events which have been associated with a sperm fusing, with an egg (whilst current clamped at a high potential) are:-

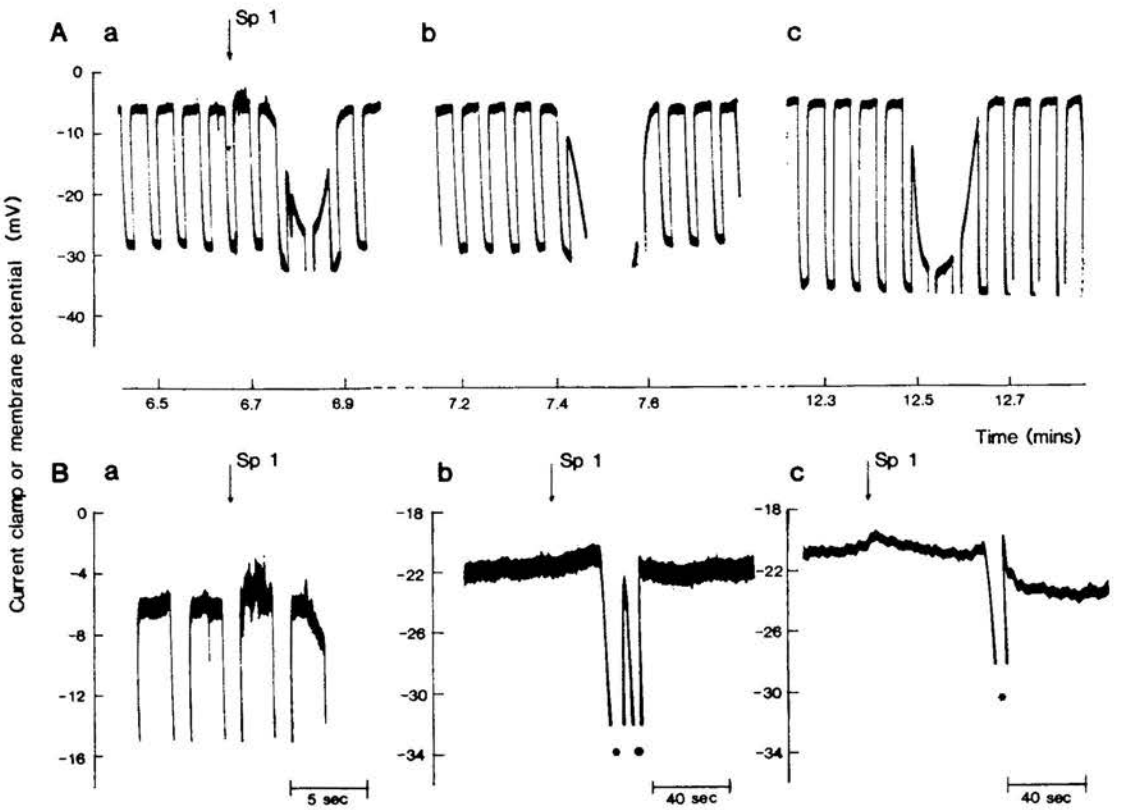
a) sperm evoked action potential
b) fsd
c) predepolarization followed immediately by a trd (the trd may even be superimposed on the predepolarization as in Figure 5.14Bd)
d) predepolarization followed shortly after by a trd spike.

These "fusion events" all involve some sort of depolarization caused by the sperm fusion. This therefore raised the question - is there any depolarization preceding the trhs which are observed when the egg is fertilized at low potentials? In order to answer this question it was necessary to analyse the results obtained during such experiments, at a high gain. The results of such an analysis are shown in Figure 5.24.

An egg impaled in Ca2K25 had a membrane potential and an input resistance of -27mV and 145MΩ. This egg was current clamped at -6mV and the recording chamber inseminated. Then 6.7 minutes later a trh was

Figure 5.24

A. Oscilloscope pictures in order of the first three trhs recorded in an egg fertilized by one sperm (fusion marked by the arrow) in Ca2K25. The fusion was accompanied by an increase in noise (Aa). Ba is a picture of the fusion shown in Aa, but at an even higher gain and faster sweep speed. The trhs in Ab and Ac were not associated with sperm fusions. Bb shows the first and second trh observed in an egg fertilized in Ca57K5. The arrow above the oscilloscope picture indicates the timing of the fusion, the asterisk and filled circle below the picture denote the first and second trh respectively. Bc is an oscilloscope picture of the first fusion in an egg bathed in Ca2K5. The asterisk denotes the first trh, and the arrow indicates the instant of sperm fusion. The responses illustrated by Aa - Ac and Ba were recorded in an egg current clamped at -6mV prior to insemination. Whereas the responses in Bb and Bc were recorded in an egg fertilized at the respective membrane potentials of -22 and -21mV. The hyperpolarizing current pulses in Aa, Ab and Ba were 0.25nA, 0.3Hz, and 1sec whereas those in Ac were 0.3nA, 0.3Hz and 1sec.


A
a
Sp 1
b
c
0
-10
-20
-30
-40
6.5
6.7
6.9
7.2
7.4
7.6
12.3
12.5
12.7
Time (mins)
Current clamp or membrane potential (mV)
B
a
Sp 1
0
-4
-8
-12
-16
5 sec
b
Sp 1
-18
-22
-26
-30
-34
40 sec
c
Sp 1
-18
-22
-26
-30
-34
40 sec

observed, but about four seconds prior to this , an increase in noise was noted (Figure 5.24Aa). Two further trhs were observed, with no further fusion of sperm (confirmed by histology to be monospermic). These trhs are shown in 5.24Ab and 5.24Ac but neither of these showed an increase in noise prior to it. The oscilloscope picture in 5.24Aa is shown in 5.24Ba at a higher gain and at a faster sweep speed.

Figure 5.24Bb shows the "top half" of two trhs recorded in an egg fertilized at a low membrane potential, bathed in Ca57K5. The sperm fused with the egg 24 seconds before the first trh (marked with an asterisk in 5.24Bb) was recorded in this egg. The second trh "started" before the first one had repolarized to the initial membrane potential. The second trh is denoted by a filled circle in Figure 5.24Bb. Both these trhs are shown in Figure 5.7, with reference to which responses at fusion were discussed at both low and high potentials in the same egg. In Figure 5.24Bb there is a small depolarization, prior to the first trh.

Similarly Fig.5.24Bc shows a "small depolarization" prior to the first trh (marked by an asterisk) observed in an egg fertilized in Ca2K5. The depolarization (in synchrony with the first sperm fusion) occurred 61 secs before the onset of the first trh. Immediately after the first trh a hyperpolarizing shift was observed. Such small depolarizations were observed to precede trhs in three other eggs.

The above results suggest that there is a very small depolarization (about 1mV) prior to the first trh recorded in an egg fertilized at low membrane potentials.

5.20 Rate of change of potential of the slow and fast responses observed during fertilization (Table 5.7)

The rate of hyperpolarizations of trhs is greater in Ca4K5 than in Ca2K5 ($p<0.05$; Fisher Behrens test). The rate of hyperpolarization of trhs was similar in Ca2K5 and Ca57K5, it being greater than that in Ca42K5.

There was no difference in the rate of depolarization of trds in Ca4K5 and Ca4K25 ($p>0.70$; two sample t test). Similarly there was no difference in the rate of depolarization of trds observed in Ca2K25 compared to Ca4K25 ($p>0.05$; Fisher Behrens test) and those in Ca2K25 compared to Ca2K25 (choline) ($p>0.05$; Fisher Behrens test).

There was no difference in the rate of depolarization of trd spikes in Ca2K25 and Ca2K25 (choline). Likewise there was no difference in the rate of depolarization of trd spikes and trds observed in Ca2K25 ($p>0.05$; Fisher Behrens test) or in Ca2K25 (choline) ($p>0.20$; two sample t test).

From these results there appears to be little effect of increasing calcium or potassium concentration in the bathing medium, on the rates of hyperpolarization (trhs) or depolarization (trds and

Table 5.7

This summarizes the rate of change of potential of the initial phase of the five types of responses observed during fertilization. The values shown denote the rate of hyperpolarization of trhs, but the rate of depolarization of the other four types of responses analysed. The thresholds of the electrically and sperm evoked action potentials are also listed. The extreme right column shows the plateau potential levels of the sperm evoked action potentials. Results are shown as mean $\pm$ SD (n=) or the actual values have been listed (in italics).

SOLUTION	Rate of change of potential (mV sec^{-1})					Threshold (mV)		Plateau potential
	trh	trd	trd spike	fsd	sperm evoked action potential	electrically evoked action potential	sperm evoked action potential	level of sperm evoked action potential (mV)
Ca2K5	7±3 (11)	8 20		5 25 27 230	216	-60±5 (9)		-56±11 (5)
Ca4K5	15±14 (21)	7±7 (9)		38±32 (28)	65±67 (11)	-56±7 (27)	-64±8 (11)	-51±14 (10)
Ca12K5						-55		
Ca42K5	3±1 (12)							
Ca57K5	8±4 (8)			11 26				
Ca2K25		13±8 (9)	28±26 (13)	57±126(20)	72±76 (9)	-61±5 (41)	-67±6 (6)	-59±8 (5)
Ca4K25	9	8±5 (6)		92 118 400		-45 -52 -55 -65		
Ca7K25			25			-48		
Ca10K25		24 29 32		4	531	-50 -50 -55 -56	-56	
Ca20K25						-62		
Ca7K40						-56		
Ca2K25 (choline)		16±16 (13)	32±18 (4)			-63±4(5)		
Ca2K25 (lithium)		10 11		55 132				

TABLE 5.7

trd spikes) of the slow responses.

The rates of depolarization of fsds in Ca4K5 and Ca2K25 were 38 ± 32mV sec^{-1}, n=28 and 57 ± 126mV sec^{-1}, n=20. When the rates of depolarization of fsds and trds in the same solution were compared there was found to be a significant difference in Ca4K5 ($p<0.01$; Fisher Behrens test) but not in those recorded in Ca2K25 ($p>0.05$; Fisher Behrens test).

The rates of depolarization of sperm evoked action potentials (i.e the rate of depolarization from the current clamp potential or the membrane potential to the threshold of the action potential) were significantly higher than that of trds recorded in the same solution (in Ca4K5 $p<0.05$, Fisher Behrens test and in Ca2K25 $p<0.05$, Fisher Behrens test). The rates of depolarization of sperm evoked action potentials were 65 ± 67mV sec^{-1}, n=11 in Ca4K5 and 72 ± 76mV sec^{-1}, n=9 in Ca2K25.

All the rates of change of potential discussed above (Table 5.7) were those measured of the initial phase of the response starting at the current clamp or membrane potential. Below is a summary of the significant differences indicated by the above results:-

a) rate of hyperpolarization of trhs is greater in Ca4K5 than in Ca2K5

b) the rate of depolarization of both types of fast responses was greater in Ca4K5 than that of trds in the same solution and

c) rate of depolarization of sperm evoked action potentials was greater than that of trds in Ca2K25.

5.21 Responses occurring immediately before or immediately after trhs or trds, which were not associated (closely in time) with sperm fusions

In 5.18, we discussed predepolarizations which occurred before trds, trd spikes, fsds and sperm evoked action potentials. But all these responses were associated with sperm fusions. Responses occurring immediately before and after trhs or trds which were not associated with sperm fusions (i.e. they were recurrent and were "triggered" by a sperm fusion several seconds before) are now discussed. These have been classified into three groups:-

a) trhs followed by after depolarizations

b) trds preceded by hyperpolarizations

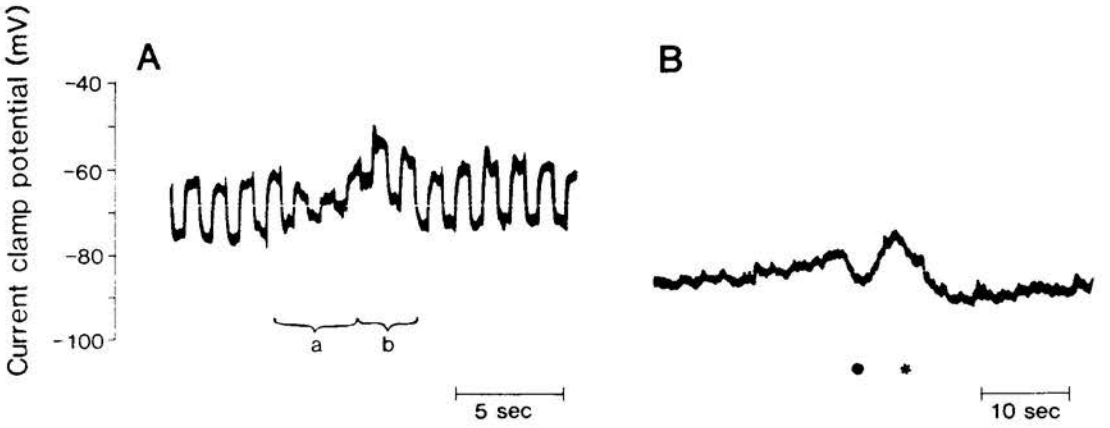
c) trds with after hyperpolarizations.

(a) Trhs followed by after depolarizations

Depolarizations were occasionally seen to follow trh responses and had been called after depolarizations. One such example is shown in Figure 5.25A. Sperm fusion in this egg (bathed in Ca4K5), elicited an action potential with a plateau. Superimposed on this plateau were 9 trhs, the fifth of which is illustrated in Fig.5.25A. The current clamp potential prior to this response (during the plateau phase) was -62mV and

Figure 5.25

Oscilloscope pictures of responses recorded in two hamster eggs fertilized in Ca4K5. A is a trh recorded with an after depolarization. The trh is marked "a" and the after depolarization is denoted as "b". Hyperpolarizing pulses were used to monitor the conductance (0.05nA, 0.8Hz, 0.8secs). Current clamp potential was -62mV. B is a trd (marked with an asterisk) preceded by a hyperpolarization (marked with a filled circle) recorded in an egg current clamped at -86mV. Neither of these responses were elicited directly by sperm fusion.


Current clamp potential (mV)
-40
-60
-80
-100
A
a
b
5 sec
B
10 sec

the reversal potential of the first phase of this response (marked "a") was -72mV. The second phase of this response (marked "b") is interpreted as being the after depolarization, and phase "a" as being the trh. Other examples of trhs with after depolarizations have been given in Figures 5.5E, Fig.5.5F, and Fig.5.9Aj.

All the illustrated examples of trhs with after depolarizations were recorded in eggs fertilized in Ca4K5. In all 13 such responses were recorded, one of these in Ca57K5, four in Ca4K5 (all of which have been illustrated) and the remaining eight in Ca2K5.

The membrane potentials (unclamped) and input resistances of the eggs in which these responses were recorded varied. For example, they were recorded in an egg bathed in Ca2K5, with a membrane potential of -18mV and an input resistance of 30MΩ. One trh with an after depolarization in this egg had a peak of -28mV, duration of seven seconds, and the membrane potential at the onset of this response was -19mV. At the other extreme was an egg bathed in Ca4K5 which on impalement had a membrane potential of -75mV and an input resistance of 250MΩ(Figure 5.9). A trh with an after depolarization (Figure 5.9Ah) in this egg, had a peak of -73mV, duration of five seconds and the membrane potential at the onset of this response was -47mV. The threshold for the electrically evoked action potential in this egg was -64mV (thus the peak of the response was more hyperpolarized than the threshold for the anode break response).

The mean duration of these trhs which were followed by after depolarizations (in Ca2K5) was 7±3 secs, n=8 which is not significantly different to the duration of trhs with no after depolarizations in Ca2K5 (6±3 secs, n=23) (as shown in Table 5.4 of this chapter).

It is possible that these after depolarizations are an attenuated manifestation of the anode break response. Although no quantitative analysis has been done to compare the rate of change of potentials of the hyperpolarizing and depolarizing phases of a trh, it is clear from the records that the rate of depolarization is slower than the rate of hyperpolarization. Thus it may be that the rate of change of potential of the depolarizing phase is insufficient to overcome the voltage inactivation of calcium channels, which is evident at low potentials (see Chapter 4).

In several trhs the peak was more hyperpolarized, than the threshold for the electrically evoked action potential in the same egg. But in these trhs an off response (equivalent to anode break excitation) in the form of a spike was not observed, in any of the above fertilized eggs. Whereas in some trd responses, a spike was observed as the initial phase (which in trds is depolarizing) "passes through" the threshold for the initiation of the spike. Again like trh responses the initial phase of trds is faster than the final decaying phase (which may be sufficient to overcome voltage

inactivation).

Another possible explanation for this after depolarization is a sodium calcium exchange mechanism which may come into operation at the end of a trh. This mechanism hypothetically would have to involve a greater influx of sodium ions than the efflux of exchanged calcium ions.

b) Trds preceded by hyperpolarizations.

Fig.5.25B is an example of a trd (marked by an asterisk) preceded by a hyperpolarization (marked by a filled circle). Another similar response is shown in Fig5.9Ai. These responses look similar to trhs followed by after depolarization, but the latter occur at membrane potentials more positive than the reversal potential for slow responses. Whereas trds preceded by hyperpolarizations were recorded at membrane potential more negative than the reversal potential for slow responses. The response shown in Fig.5.25B was recorded in an egg bathed in Ca4K5, and current clamped at -90mV. Three such responses were recorded in this egg, the second one of which is illustrated in Fig.5.25B. The first response occurred 120 seconds after imposing the current clamp, and the second and third response occurred at 100 and 197 seconds after this respectively. These responses were therefore cyclic in nature, and cannot be interpreted as being fsds followed by trds. Furthermore they were not associated with sperm fusions and cannot therefore be

called fsds.

Five such responses have been observed in two eggs, both of which were fertilized in Ca4K5. The current clamp potential at the onset of the response was -89±3mV (n=5), at the peak of the hyperpolarization was -99±4mV (n=5) and at the peak of the trd it was -83±2mV (n=5). The duration of the hyperpolarizing phase was 4±0.4 seconds (n=5) and that of the trd phase was 4±0.7 seconds (n=5). The duration of the whole response was 9±1 seconds (n=5) as compared to 8±2 seconds (n=31) for trds with no pre-hyperpolarizations.

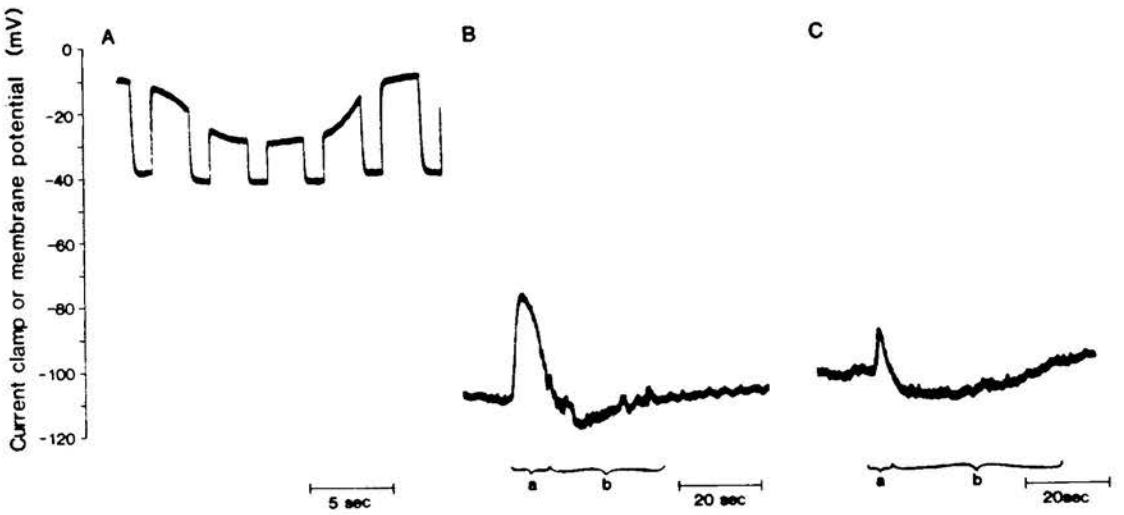
These five results may therefore be indicative of "a dual mechanism" responsible for trhs which is not usually discerned. It may also be a pointer to the fact that there is a great variety of responses which can be recorded in these eggs during fertilization. It is also curious that the two eggs in which these five responses were recorded were the first two eggs fertilized on that particular day. The membrane potentials and input resistances of these two eggs were -75mV, and 250MΩ and -45mV, 220MΩ.

c) Trds with after hyperpolarizations.

Only one fertilization throughout the course of this study was attempted in Ca2K25 (lithium). The membrane potential and input resistance of that egg was -7mV and 70MΩ respectively. The egg was fertilized, but no electrical event accompanied the first sperm

Figure 5.26

Responses recorded during the monospermic fertilization of a zona-free hamster egg bathed in Ca2K25 (lithium). A is a trh recorded in this egg the reversal potential of which was found to be -43mV. Hyperpolarizing pulses were used to monitor the conductance throughout this response (0.5nA, 1.2sec, 0.3Hz). B and C are two trd responses with after hyperpolarizations recorded in the same egg after it had been current clamped to -110mV. In B and C, "a" refers to the trd and "b" denotes the after hyperpolarization.


Current clamp or membrane potential (mV)
0
-20
-40
-60
-80
-100
-120
A
B
C
5 sec
a
b
20 sec
a
b
20sec

fusion. Two trhs were then recorded, the first of which is shown in Figure 5.26A (the reversal potential for which was -43mV). The reversal potential of the second trh, which is not shown was -37mV. The egg was then current clamped at -110mV, at which potential two trds were recorded (shown in Figure 5.26B and 5.26C). Both of these had prominent after hyperpolarizations (they were not associated with sperm fusions - the egg was subsequently confirmed by histology to be monospermic). The peak potential of the first was -76mV, amplitude was 34mV and it's duration was 9 seconds. Similarly the peak potential of the second was -93mV, amplitude was 13 mV and the duration was five seconds. The durations of the after hyperpolarizations were 20 seconds and 40 seconds respectively. The trds have been denoted by "a" and the after hyperpolarizations by "b". Such after hyperpolarizations were only observed in this experiment, they were not recorded in eggs fertilized in other solutions.

It is interesting that there was no evidence of a biphasic event at low potentials (e.g. in Fig.5.26A).

5.22 A comparison of the thresholds of the sperm and electrically evoked action potentials (Table 5.7)

The threshold of the sperm evoked action potential was more hyperpolarized than that of the electrically evoked action potential in both Ca4K5 ($p<0.01$; two sample t test) and Ca2K25 ($p<0.05$; two sample t test).

In five eggs fertilized in Ca4K5 it was possible to measure the thresholds of both the sperm evoked and the electrically evoked action potentials. The thresholds of the electrically evoked action potentials were -64, -60, -60, -64 and -50mV and that of the sperm evoked action potentials were -56, -56, -64, -64 and -58mV respectively, i.e. in two of these five eggs the threshold of the sperm evoked action potential was more hyperpolarized than that of the electrically evoked action potential.

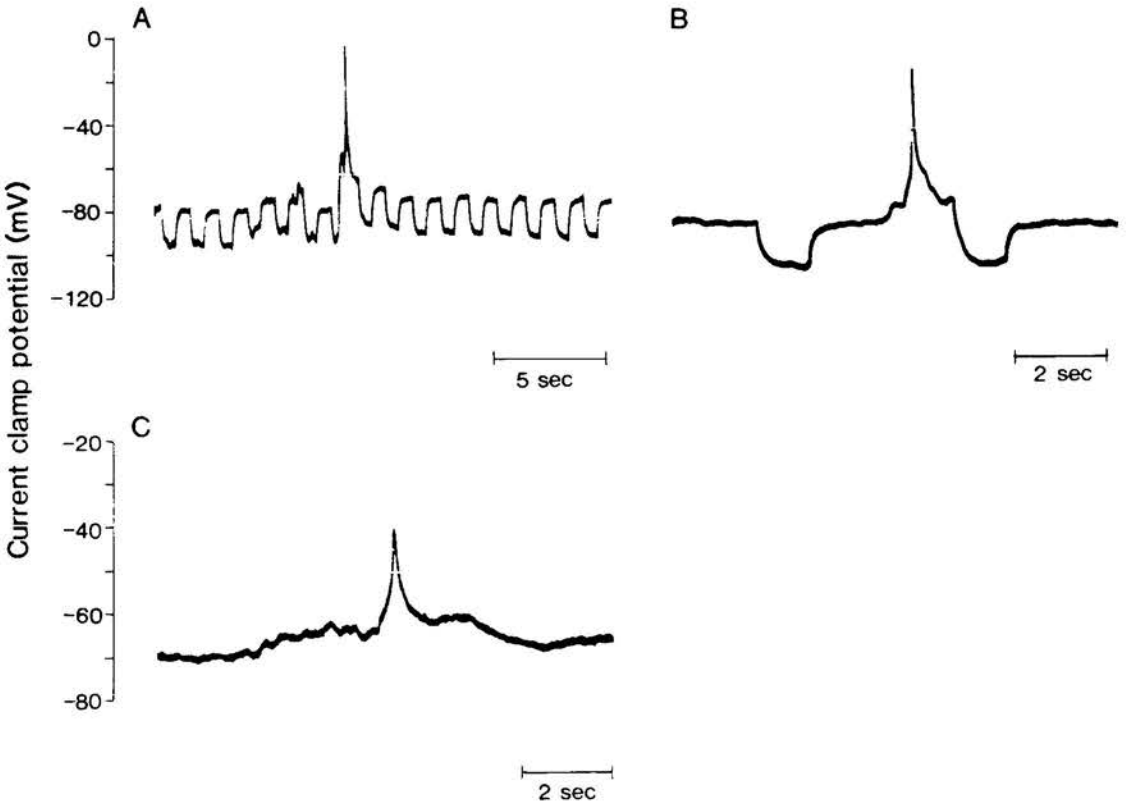
Similarly the thresholds of the electrically evoked action potentials in six eggs bathed in Ca2K25 were -65, -60, -64, -55, -60, and -64mV and that of the sperm evoked action potentials were -76, -68, -70, -56, -68 and -64mV respectively, i.e. in five of these six eggs the threshold of the sperm evoked action potential was more hyperpolarized than that of the electrically evoked action potential.

The thresholds of the electrically evoked action potentials were similar in Ca2K5 (-60 ± 5mV, n=9), Ca2K25 (-61 ± 5mV, n=41) and Ca2K25 (choline) (-63 ± 4mV, n=5).

No significant difference was found in the thresholds of the electrically evoked action potential in Ca2K5 and Ca4K5 ($p>0.1$; two sample t test). But comparing the four values for the threshold of electrically evoked action potentials in Ca10K25 (-50, -50, -55 and -56mV), with the results available in

Figure 5.27

Oscilloscope pictures of brief action potentials recorded in three eggs. The bathing solution for the response shown in A was Ca2K5, for B it was Ca4K5 and for C it was Ca2K25. The hyperpolarizing pulses in A were 0.07nA, 0.7secs and 0.8Hz and in B were 0.1nA, 1.2secs and 0.2Hz.


A
B
C
0
-40
-80
-120
-20
-40
-60
-80
Current clamp potential (mV)
5 sec
2 sec
2 sec

Ca2K25, one may conclude that elevating calcium concentrations in the bathing medium, does in fact make the threshold, more positive.

5.23 Plateau potential levels of sperm evoked action potentials (Table 5.7)

Sperm evoked action potentials occasionally plateaued and did not return to their initial membrane or current clamp potential values. In such "permanent plateaux", any further sperm evoked responses were superimposed on these plateaux. Illustrations of these have been given in Figures 5.3, 5.4, 5.12, 5.13 and 5.19. The levels of these plateaux (Table 5.7) were -56±11mV, n=5 in Ca2K5; -51±14mV, n=10 in Ca4K5 and -59±8mV, n=5 in Ca2K25. These were not significantly different from each other.

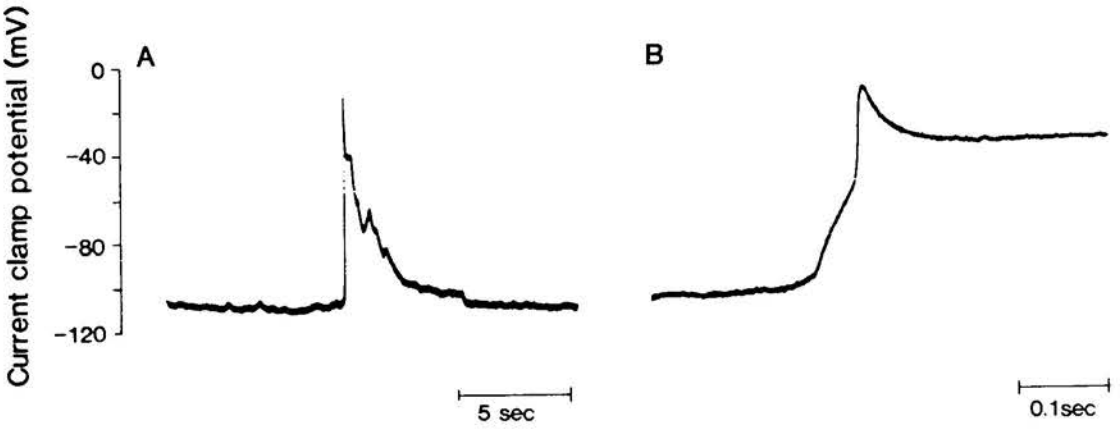
5.24 Brief action potentials

On three occasions brief action potentials were observed which were not accompanied by sperm fusions (Figure 5.27). It is possible that the event marked "c" in Figure 5.23B is also a brief action potential. One brief action potential was observed in an egg fertilized in Ca2K5, one in Ca4K5 and another in Ca2K25 (a different egg in each of the three different solutions).

The spike peaks of these brief action potentials were -6mV (Ca2K5 - Figure 5.27A), -16mV (Ca4K5 - Figure 5.27B) and -42mV (Ca2K25 - Figure 5.27C).

Figure 5.28

A is a spontaneous action potential observed in zona-free hamster egg current clamped at -109mV. B is a faster sweep speed picture of the action potential shown in A.


Current clamp potential (mV)
0
-40
-80
-120
A
5 sec
B
0.1sec

The durations of these brief spikes was about one second, but one of them was superimposed on a small depolarization lasting six seconds (Figure 5.27C). Further examples of brief action potentials are also described in Appendix A.

5.25 Spontaneous action potentials.

Occasionally spontaneous action potentials were observed in eggs. These were not due to movement of the sperm attached to the egg, since they could be recorded in eggs with no sperm attached. Histology showed that eggs in which spontaneous action potentials were recorded were not parthenogenetically activated. It was possible to fertilize an egg, after a spontaneous action potential had been recorded in it.

In recordings from 146 eggs described in this chapter, in only three eggs was a spontaneous action potential observed (although many were observed in a separate series of experiments, described in Appendix A). Due to their infrequent nature they were not amenable to study. One of these spontaneous action potentials is shown in Figure 5.28A. Figure 5.28B is the same action potential shown in Fig.5.28A, at a faster sweep speed. Its threshold was -60mV, its peak potential was -10mV and its duration was 5 seconds (much shorter in duration than the sperm evoked action potentials).

5.26 Discussion

Fertilization of low membrane potential eggs

Homologous fertilizations of low membrane potential hamster eggs in the present study indicated many similarities to the results obtained by Igusa and Miyazaki (1983):-

a) The frequency of trhs was increased, by increasing the external calcium. For example in Fig.5.1 the egg was bathed in Ca2K5, and intervals between successive trhs ranged from 1.2 to 1.9 minutes. Whereas in Fig.5.2 the egg was bathed in Ca42K5, and the intervals were no larger than about 0.5 minutes.

b) The trhs were superimposed on a gradual hyperpolarization of the membrane potential.

c) The frequency of trhs increased with more negative current clamp potentials.

But Igusa and Miyazaki (1983) reported that the reversal potentials of trhs (in solutions containing 0.8-12mM calcium) lay in the range -86 to -83mV. In the present study the reversal potentials of trhs recorded in Ca2K5 were more positive than this, i.e. -69 ± 9mV (n=40, Table 5.5).

Although no significant difference was found in the reversal potentials of trhs recorded in Ca2K5 and Ca4K5 the mean was more negative in the latter. It is suggested that the measures of reversal potential in Ca4K5 are a better approximation to the true reversal potential of slow responses, because the input resistances of the eggs in the latter are higher. It

is therefore assumed that they are less influenced by the leakage pathway. Input resistances of eggs in Ca4K5 were significantly higher than those in Ca2K5 (see 5.8). This is illustrated as follows:-

let E = reversal potential of the response caused by an increase in the potassium conductance

Er = membrane potential at the onset of the response (let it be -30mV, in this example)

Ek = potassium equilibrium potential, which is assumed to be the peak of the response (let it be -80mV, in this example)

Gr = conductance of the membrane prior to the response

Gk = conductance of the membrane at the peak of the response

then

$$E = \frac{Er\ Gr + Ek\ Gk}{Gr + Gk} = \frac{Er + Ek\ \ (Gk/Gr)}{1 + (Gk/Gr)}$$

if Gk = Gr, as might be the case if the input resistance of the egg was low, due to a large "leak" then

$$E = \frac{-30 + (-80)\ 1}{2} = -55mV$$

i.e. in this situation the reversal potential is calculated to be -55mV.

If on the other hand Gk = 100 x Gr, i.e. the input resistance of the egg before the response, is high and the conductance increase at the peak of the response is

100 fold, then

$$E \triangleq \frac{-30 + (-80)\ 100}{101} = -80mV$$

Therefore, theoretically the higher the input resistance of the egg then the smaller is the difference between the measured and the true reversal potential of the response.

The more negative peak potential in Ca4K5, may also be due to the higher input resistances and membrane potentials of eggs, bathed in this solution (5.8 and Table 5.2) compared to Ca2K5.

Cytosolic calcium increases following sperm fusion

There were several indications from the results discussed in this chapter that the calcium increased immediately after a sperm fusion:-

a) a slow response immediately following a sperm fusion in an egg current clamped at a high potential was longer lasting than subsequent slow responses (Figs.5.15, 5.16 and 5.21). This may be because the level of cytosolic calcium remains above the threshold for activating the calcium activated potassium conductance, for a longer time. This would imply a limited buffering capacity (including the sequestration of calcium by intracellular stores) for calcium and/or a limited extrusion rate of calcium.

b) in eggs current clamped at high potentials and bathed in high potassium solutions a sperm fusion

associated with a trd spike had a reduced spike height (Fig.5.21). This would be explained by a more negative equilibrium potential for calcium because of an increase in the cytosolic calcium.

c) the spike peak of anode break responses became more negative, following a sperm fusion. For example in Fig.5.19A, the spike peak hyperpolarized by about 27mV in about eight seconds after a sperm fusion. From Nernst one would expect at 34°C, a 30mV shift in the calcium equilibrium potential (in the hyperpolarizing direction) for a tenfold change in cytosolic calcium. Hence in the example illustrated there might be nearly a tenfold increase in cytosolic calcium after about eight seconds following fusion.

d) occasionally sperm fusions with eggs current clamped at high potentials were associated with sperm evoked action potentials. Similar sperm evoked responses have been recorded in eggs bathed in a solution resembling oviducal fluid and in a solution with a low sodium concentration. Since these eggs have voltage dependent calcium channels but apparently no sodium channels (Okamoto et al., 1977; Georgiou et al., 1984; Bland, Bountra, Georgiou, House & Martin, 1984 - a copy of this is in Appendix D; Chapters 3 and 4) the sperm evoked action potential must arise from a voltage-gated influx of calcium ions. Such a sperm evoked action potential was observed in an egg with a high membrane potential (Fig.5.9).

e) in some eggs current clamped at high potentials, sperm fusions occurred in synchrony with fsds. But such responses were observed in Ca2K25 (choline), and must therefore be indicative of a calcium influx (Fig.5.16). The reversal potentials of fsds, indicate that such depolarizations cannot be due to the movement of any other ion. In many experiments, eggs were deliberately current clamped at very large negative potentials, e.g. -130 to -140mV. This was in order to increase the driving force for calcium influx, and hence enhance the amplitude of any depolarization which might accompany a sperm fusion. Also this would partly compensate for any loss of potential which might occur between the time of sperm addition to the chamber and sperm fusion with the egg (due to a decrease in the seal resistance between the electrode tip and the egg membrane). It is therefore possible that in some experiments had the current clamp potential been more positive, then such fsds might have given rise to sperm evoked action potentials. This would have happened only if the peak of the fsd in a given egg was more positive than the threshold for the sperm evoked action potential in that egg (e.g. the first fusion in Fig.5.5).

Ionic basis of the sperm evoked response

The ionic basis of the sperm evoked response has not yet been examined. The spike peaks of sperm evoked action potentials were more negative than those of

electrically evoked action potentials recorded in eggs bathed in Ca2K25 (5.14). Although no significant difference was observed in such measurements made in eggs bathed in Ca4K5. This difference in spike peaks observed in Ca2K25, signifies the presence in the fertilized egg of a source of outward current through some pathway not present or less effective in unfertilized eggs. It is conceivable that such a mechanism of producing outward current at low membrane potentials, would limit the height of the action potential, and might also influence the level of the plateau phase of the response. The fact that no significant difference was noted in spike peaks of the two types of action potential in Ca4K5 may be an indication that this outward current is not an outward potassium movement.

No significant difference was observed in the plateau potential levels of sperm evoked action potentials, recorded in Ca2K5, Ca4K5 and Ca2K25. But these comparisons should be treated with a little caution since the number of results available were only 5, 10 and 5 respectively (5.23 and Table 5.7). Furthermore such potential levels will be dependent on the seal resistance and the steady current being passed through the electrode to current clamp the egg, as well as the input resistance of the egg. In the face of these cautionary notes, a hypothesis worthy of experimental test is that a prominent rise in the

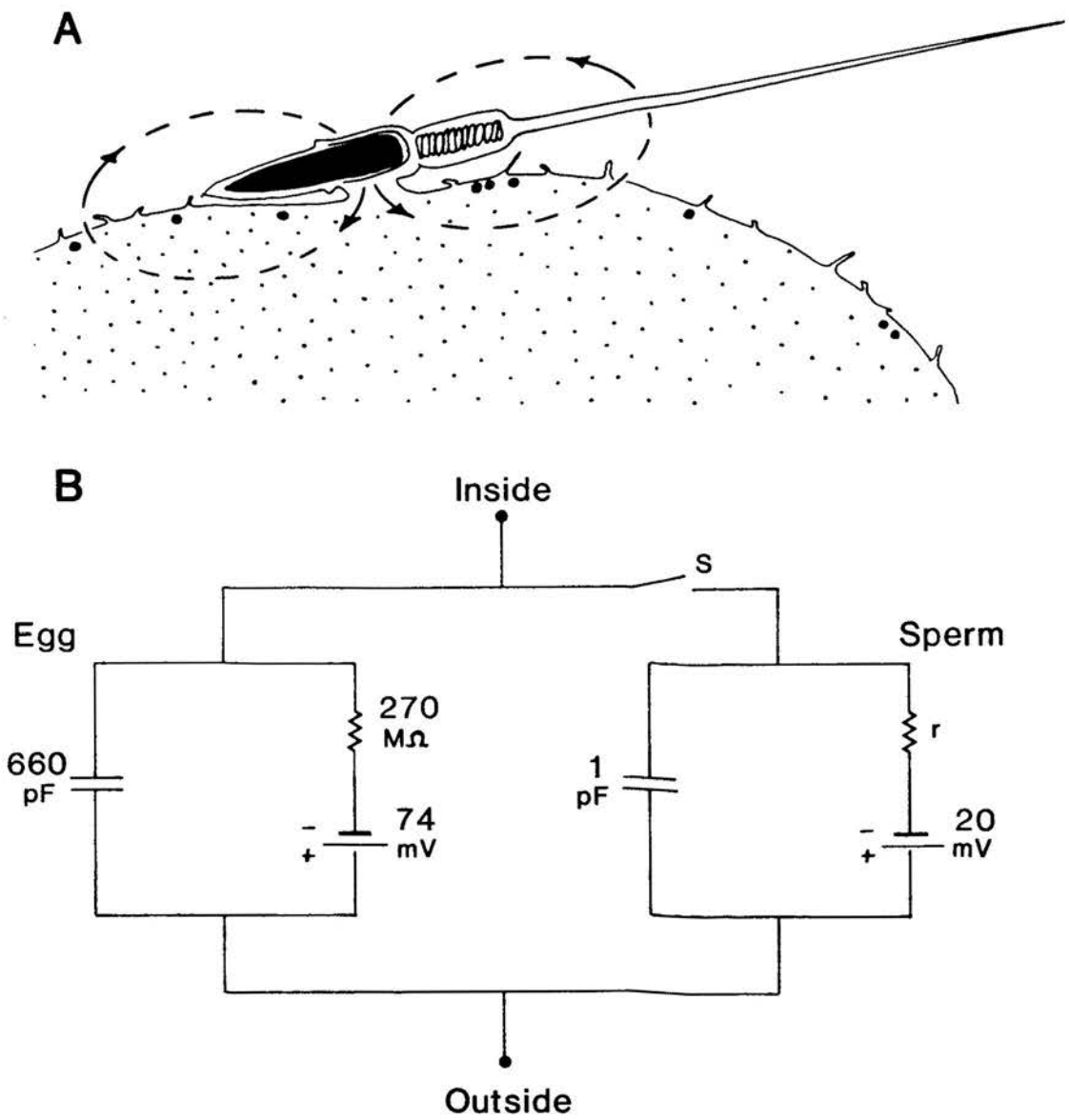
cytosolic calcium occurs after sperm egg fusion and opens the potassium channels in the egg's membrane (Georgiou et al., 1983; Chapter 3). Consequently an outward potassium current would flow and cause repolarization. It might be that the sperm head is a source of calcium which acts as a trigger for further calcium release within the egg as has been suggested for the fertilized sea urchin egg (Baker, 1980; Jaffe, 1980). This possibility is discussed a little further in the discussion of Chapter 6.

Sperm egg fusion causes a depolarization of eggs with high membrane potentials or those current clamped at high potentials (Figs.5.7 and 5.9). Whereas in eggs fertilized at low membrane potentials, sperm fusion is not usually seen as a depolarization (Fig.5.7) unless the recording is made at a very high sensitivity and an appropriate speed (5.19 and Fig 5.24). Only on one occasion was a small depolarization observed to accompany a sperm fusion, when the recording was made on a pen chart (Fig.5.1).

At the instant of fusion the plasma membrane of the spermatozoon is inserted into that of the egg (Fig.5.29A). The insertion process has been analysed (Miyazaki & Igusa, 1981) by the application of an equivalent circuit (Miyazaki, 1979) commonly used to describe synaptic potentials (e.g. Ginsborg, 1973). Figure 5.29B shows such an analogue circuit in which closing the switch S corresponds to fusion of sperm and egg. Values for the membrane potential and input

Figure 5.29

A. Diagram illustrating the possible current flow (broken lines) at the instant of fusion between a sperm and an egg. B. Equivalent electrical circuit used to analyse the electrical responses recorded during fertilization (see text). At fusion the switch "S" is closed.


A
B
Inside
S
Egg
Sperm
270
MΩ
660
pF
74
mV
1
pF
r
20
mV
Outside

resistance of a "high potential egg" have been taken as the means of values recorded in Table 4.1 (data on the five high potential eggs observed in the study). These values are -74mV and 270MΩ (denoted as ϵ and R respectively) respectively. The capacitance of a zona-free hamster egg has been taken as 660pF (Georgiou et al., 1984). Little is known about the electrical properties of sperm. Miyazaki and Igusa (1981) assumed that the battery (e) operating across the sperm membrane at fusion would be -20mV. A rough estimate of the sperm's surface area (about 10^{-6} cm^2) suggests that the capacitance would be about 1pF. An unconfirmed estimate of the input resistance of bull spermatozoa is 20MΩ (Lindemann & Rikmenspoel, 1971). This is probably an underestimate. In the absence of resistance measurements on hamster sperm, the resistance has been called "r".

According to the equivalent circuit (Fig.5.29B) the rising phase of the depolarization (ΔV) occurring at fusion, i.e. when S closes, is given by:

$$\Delta V = \frac{R}{R+r} \quad (e-E) \; [1-\exp(-t/\tau)] \qquad (1)$$

where τ is the time constant (effective capacitance multiplied by the effective resistance) of the sperm and egg in parallel. The steady value of the depolarization ΔV attained after fusion (in the absence of an action potential) is given by:

$$\Delta V = \frac{R}{R+r} \quad (e-E) \qquad (2)$$

Since the mean value of the threshold of sperm evoked action potentials in this study was -64mV, then the minimum size of the sperm induced depolarization required to elicit a sperm evoked action potential is 10mV (because membrane potential is taken as being -74 mV). Therefore given that ΔV must equal or exceed about 10mV to evoke an action potential then according to equation (2) the maximum value of r is about 1190MΩ. Since

$$10\text{x}10^{-3} = \left[\frac{270\text{x}10^{6}}{([270\text{x}10^{6}]+r)}\right](-20-[-74])\text{x}10^{-3}$$

Effective resistance of sperm and egg in parallel

$$= \frac{(1190\text{x}10^{6}) \text{ x } (270\text{x}10^{6})}{(1190 + 270) \text{ x } 10^{6}}$$

$$= 220\text{M}\Omega.$$

Effective capacitance of sperm and egg in parallel

$$= 660 + 1$$

$$= 661 \text{ pF}$$

$$\therefore \tau = (661\text{x}10^{-12}) \text{ x } (220\text{x}10^{6})$$

$$= 145\text{ms}$$

A value of 1190MΩ for the sperm's resistance means that its specific membrane resistance would be about $1190\Omega\text{cm}^2$, assuming surface area = 10^{-6}cm^2 (see above). Assuming that the specific capacitance of hamster egg membranes is $1.0\mu\text{F cm}^{-2}$, then the surface area of the egg is $6.6\text{x}10^{-4}$ cm^2. If the surface area is calculated

from the diameter, then it is underestimated, because of the presence of numerous microvilli on the surface. (Such a calculation results in a surface area of $2.0 \times 10^{-4} cm^2$, if the diameter is 80μm; Georgiou et al., 1984). Therefore the specific resistance of the egg is equal to:-

$$(270 \times 10^{6}) \times (6.6 \times 10^{-4})$$

$$= 178200 \simeq 180{,}000\ \Omega cm^2$$

Thus the calculated specific membrane resistance of the sperm membrane is substantially less than that of the egg membrane, on this basis.

Estimates of the membrane potential of bull and guinea pig sperm are -6mV (Lindemann & Rikmenspoel, 1971) and +13mV (Rink,1977) respectively. If e is assumed to be 0mV then the maximum value of r for a 10mV sperm evoked depolarization becomes 1730MΩ and that for τ is 154ms.

In the case where E equals -26mV and R equals 150MΩ (the mean membrane potential and mean input resistance of eggs bathed in Ca2K5 in this study) then the depolarization ΔV predicted by equation (2) is about 0.7mV for r = 1190MΩ and e = -20mV. When r = 1730MΩ and e = 0mV the expected depolarization would be 2.1mV. The value for τ determining the rising phases of these depolarizations would be about 90ms. Detection of such small depolarizations in eggs fertilized at low potentials, is made more difficult in some cases by the masking effect of trhs (Fig.5.24). It is possible that

small depolarizations caused by sperm fusions at high potentials may be masked by trds or trd spikes. But at large negative potentials the driving force for calcium influx is larger than at low potentials, hence any depolarization at high potentials would be larger for a given calcium permeability. Such small depolarizations occurring before trds or trd spikes, have been observed to accompany sperm fusions at high current clamp potentials. These have been called pre-depolarizations, in the present study.

The interval beween the depolarization caused by the first sperm fusion and the first slow response in any egg was variable. For example in Fig.5.23A it is possible to distinguish between the depolarization caused by the fusion, and the trd spike which followed it about 10 seconds later. Similarly in Fig.5.19A, the first slow response occurred about 16 seconds after the sperm fusion.

The fusion of a sperm to an egg with a low membrane potential is followed by a trh (Miyazaki & Igusa, 1981, 1982). Since the voltage dependent calcium channels are inactivated at low membrane potentials (Okamoto et al., 1977; Chapter 3 and 4) the absence of a sperm evoked calcium action potential in those experiments is not surprising. However, the mechanism underlying the sperm evoked depolarization at high membrane or current clamp potentials could still operate at low potentials although not producing a conspicuous depolarization (see above). The profound

differences in the potential changes evoked by sperm fusions in eggs at different potentials could be reconciled if this mechanism involves an increase in calcium permeability. This idea can be expressed as follows. Sperm egg fusion causes an increase in the calcium permeability of the egg membrane, which produces a small depolarization in low potential eggs and a larger depolarization in eggs with high membrane potentials or those current clamped at high potentials. In the latter group, if the depolarization is large enough to reach threshold then a sperm evoked action potential is elicited. The increased calcium permeability would cause an increase in the cytosolic calcium. In eggs with sperm evoked action potentials this increase in the cytosolic calcium would be larger. The increase in cytosolic calcium may activate a potassium permeability which may:-

a) in low potential eggs cause trhs

b) in high potential eggs or those current clamped at high potentials may cause trds

c) be partly responsible for the repolarization of the sperm evoked action potential

d) cause trhs or trds superimposed on the plateau of a sperm evoked action potential or an fsd.

Apparently sodium entry into the egg is not crucial for the sperm evoked depolarization (5.7) whereas an influx of sodium occurs during fertilization of invertebrate eggs (Hagiwara & Jaffe, 1979). If the

membrane potential of eggs is high (i.e. more negative than threshold) in high potassium solutions, then another interesting possibility is raised (this situation is analogous to current clamping eggs at high potentials, in high potassium solutions). If the sperm releases calcium into the egg's cytosol, or a substance which promotes calcium release from one or more stores within the eggs (Jaffe, 1980; Baker, 1980) then the increase in the cytosolic calcium produced may increase the potassium permeability of the egg. This would cause a depolarization in high potential eggs (and in those current clamped at high potentials) and possibly elicit a trd spike response. In eggs with potentials more positive than the potassium equilibrium potential trhs would be observed. The above two schemes are not mutually exclusive.

Igusa et al (1983) have proposed that recurring hyperpolarizations are caused by secondary release of intracellular calcium in the egg. This explanation is consistent with the transient light flashes recorded from aequorin-labelled fertilized mouse eggs by Cuthbertson et al. (1981).

Apparently the influx of calcium ions causing the upstroke of the sperm evoked action potential is not quantitatively sufficient by itself to initiate fertilization because electrically evoked action potentials do not activate the egg (Fig.2.6). Indeed a change of membrane potential by itself is not sufficient to cause fertilization because fertilization

occurs in eggs with low and high potentials in which different voltage transients follow sperm egg fusion. It remains possible, however, that the calcium influx during the upstroke of the sperm evoked action potential acts as a trigger for the exocytosis of the substances in the egg's cortical granules. In fertilized hamster eggs (Barros & Yanagimachi, 1971) or electrically stimulated unfertilized eggs (Gwatkin et al., 1973) the material released into the perivitelline space renders the zona pellucida impenetrable to sperm. Those findings are consistent with the hypothesis that the calcium action potential causes the release of the cortical granule contents because zona-free hamster eggs readily becomes polyspermic (Yanagimachi, 1972b; Hanada & Chang, 1972). The present results indicate that there is no fast block to polyspermy in the zona-free hamster egg . This conclusion might not apply to eggs of other mammals. In the mouse egg, for example, the block to polyspermy is exerted at both the egg's plasma membrane and the zona pellucida (Wolf, 1978). The absence of a marked change in membrane potential of zona-free mouse eggs at fertilization has been taken as evidence, however, that there is no electrical block to polyspermy at the egg membrane (Jaffe, Sharp & Wolf, 1983; 1.20).

The ionic basis of the fertilization responses recorded in zona-free hamster eggs require further

investigation

In the present study there were indications that trhs may not be simply due to a calcium activated potassium conductance. There may be other mechanisms, e.g. sodium calcium exchange, which facilitates the repolarization during such a response. Pointers to this include:-

a) occasionally trhs were followed by after -depolarizations

b) reversal potential of trhs and trds (see 5.13 and Table 5.5)

c) many more fertilizations need to be performed in sodium free, lithium containing solutions (5.21c).

Other questions raised are:-

a) results of the present study indicate that the thresholds of sperm evoked action potentials are more negative than those of electrically evoked action potentials - what is the basis of this? (see 5.22).

b) what causes the decrease in resistance after fertilization? (see 5.8)

c) is there any difference in the reversal potential of a slow response superimposed on a plateau (of an fsd or a sperm evoked action potential) and that occurring prior to a plateau? (Fig.5.19).

Heterologous fertilizations of zona-free hamster eggs with mice sperm

Only five hamster eggs were fertilized in the present study with mice sperm. Trhs and trds were not

observed in any of these, which may be because the eggs were current clamped close to the reversal potential of these responses. Furthermore one egg which was fertilized by two sperm, showed no electrical event at the time of fusion or thereafter. Igusa et al (1983) observed that zona-free hamster eggs allowed multiple entries of mice sperm. Such fertilizations in their study showed a hyperpolarizing shift of the resting potential from about -25mV (membrane potential of unfertilized hamster eggs) to about -60mV. Superimposed on this, were several trhs. Sperm fusion was accompanied by hyperpolarizing steps (3-7mV) occurring 4-50 seconds ahead of a cessation of sperm motion (see 1.21). These workers noted that occasionally transient depolarizations appeared sporadically, whilst the membrane potential of the fertilized hamster egg was about -60mV. These may have been associated with further sperm fusions, although they present no evidence for this. If this was the case then it is possible that these transient depolarizations would have given rise to sperm evoked action potentials, had the eggs been current clamped at potentials below threshold.

Possible relevance of high potassium solution to fertilization

Some fertilizations were performed in the present study in high potassium solutions. This is because

such high concentrations of potassium have been detected in the micro-environment of the pre-implantation mouse embryo (Borland et al., 1977). The significance of this is not known. An alteration in the external potassium may change the concentration of intracellular potassium. In other preparations, an alteration of the latter has been shown to affect the activity of glycolytic enzymes (Scholnick, Lang & Racker, 1973) and macromolecular syntheses (Rozengurt & Heppel, 1975). High potassium concentrations however are not essential for fertilization or cleavage in the mouse and hamster, since all chemically defined media used, contain potassium concentrations which resemble those of serum, i.e. about 5mM (Biggers et al., 1971).

Although we should not equate incorporation and fusion, it is interesting to note that Vergara and Latorre (1983) found it difficult to incorporate calcium activated potassium channels (isolated from rabbit muscle) into planar bilayers if the concentration of potassium in the surrounding medium was less than 100mM. This may be an indication that higher extracellular potassium concentrations facilitate fusion of membranes (in the present study those of sperm and egg).

When acrosome reacted guinea pig sperm were added to zona-free guinea pig eggs, in a medium lacking potassium, the sperm were unable to penetrate the vitellus (Rogers, Ueno & Yanagimachi, 1981). The minimum concentration of potassium required for

successful fertilization was between 1 and 2 mM. Concentrations of potassium in the bathing medium between 3 and 5mM gave 100% fertilizations. Mahi and Yanagimachi (1978) demonstrated a potassium requirement at the level of membrane fusion, by the fact that dog sperm in the absence of potassium attached to zona-free eggs but could easily be removed by pipetting. Whereas sperm could not be removed by pipetting in the presence of potassium. Rogers et al (1981) have suggested that the potassium requirement for fusion of guinea pig sperm to the vitellus may be due to a stimulation of metabolism by potassium, which may effect the maintenance of the transmembrane potential and fusion.

Does microelectrode insertion mimic sperm entry?

The progressive increase in membrane potential and resistance observed after impalement of zona-free hamster eggs (see 4.1) might be due to processes initiated by electrode insertion itself, rather than to an improved sealing of the egg membrane around the microelectrode tip, as suggested in chapter 4. It could therefore be that a rise in potential and resistance is caused by sperm fusion and that the insertion of a microelectrode mimics this. A rise in potential and resistance is observed after impalement of invertebrate eggs (e.g. Chambers & de Armendi, 1979). Evidence from sea urchin eggs based on

intracellular recordings from unfertilized and fertilized eggs, on unidirectional flux measurements in unfertilized eggs (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979; Whitaker & Steinhardt, 1982) and on extracellular recordings of "action currents" (Whitaker & Steinhardt, 1983) at fertilization indicates that microelectrode insertion does not mimic sperm entry.

Since no workers have reported a rise in resistance after fertilization (1.14-1.22) it seems unlikely that the sealing process described in Chapter 4 is an indication that the egg has been activated by microelectrode penetration.

Uehara and Yanagimachi (1977) were able to activate hamster eggs by making several piercing movements with glass needles of diameters in the range 3-5 μm. Since the piercing movement amounted to pushing the needle through the egg until it emerged at the other side, it is unlikely therefore that the gentle insertion of fine tipped microelectrodes can activate mammalian eggs. These authors noted that needles with diameters less than 2 μm were ineffective at causing activation. Furthermore, Fulton and Whittingham (1978) have reported that insertion of micro pipettes with fine tips (less than 0.5μm) did not activate mouse eggs.

CHAPTER 6 INTRACELLULAR RECORDINGS DURING HOMOLOGOUS FERTILIZATIONS OF ZONA-FREE HAMSTER EGGS BATHED IN CALCIUM-FREE SOLUTIONS, CONTAINING SUBSTITUTED MULTIVALENT IONS

In the previous chapter, results of fertilizations performed in normal and high potassium solutions were described. But in all these solutions the only divalent ion present was calcium (other than 1.2mM magnesium chloride), the concentration of which was varied. Since it is believed that an influx of calcium ions plays a major role in the responses described, it was necessary to perform similar experiments in solutions containing no added calcium, but containing another multivalent ion instead. Four species of ion were used to substitute for the calcium. They were:-

a) Barium ions (barium chloride), i.e. Ba^{2+}.

b) Lanthanum ions (lanthanum nitrate), i.e. La^{3+}

c) Magnesium ions (magnesium chloride), i.e. Mg^{2+}

d) Strontium ions (strontium chloride), i.e. Sr^{2+}.

All the experiments described in this chapter are homologous fertilizations of zona-free hamster eggs, bathed in "calcium-free" solutions substituted by one of the above ions. The concentration of the ion used is indicated in the same way as the concentration of calcium was in the last chapter.

Experiments were similarily performed in either normal or high potassium solutions.

Two major difficulties were noted in these experiments. Firstly, it was difficult to current clamp the eggs in barium and magnesium substituted calcium-free solutions, at high potentials because the eggs decreased in resistance quickly (compared to calcium containing solutions). Secondly, the sperm

introduced into the recording chamber (which contained the multivalent ion substituted, calcium-free solution) did not survive as well as they did in calcium containing solutions.

Since the solution in which sperm were capacitated contained 1.8mM calcium (modified Tyrode), addition of sperm to the recording chamber would inevitably introduce some calcium (to the calcium-free medium bathing the egg). The amount of calcium introduced was dependent on the volume of sperm added. The final concentration of calcium in the medium (dependent also on the volume of the bathing medium) was never more than 70μM.

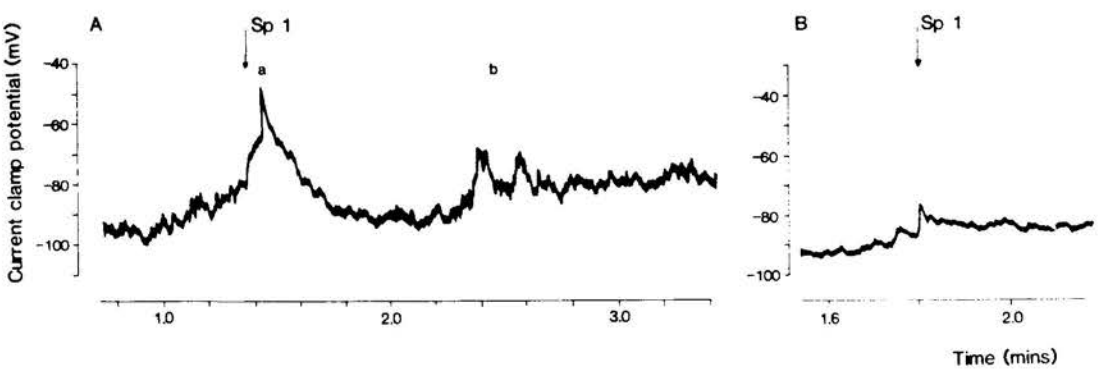
6.1 Fertilizations in calcium-free, barium substituted solutions

During the course of this study, six hamster eggs were fertilized in calcium-free, barium substituted solutions (all containing high potassium, i.e. 25mM). Five of these were fertilized in a solution containing 5mM barium chloride, i.e. Ba5K25, and the remaining one in Ba10K25. The egg fertilized in Ba10K25 had a membrane potential of -61mV and an input resistance of 340MΩ (discussed in Chapter 4). The threshold for the electrically evoked action potential in this egg was -56mV, hence by definition it was a high potential egg.

Of the six eggs fertilized, in only two were electrical events observed to accompany sperm fusion.

Figure 6.1

A. The electrical events recorded in an egg fertilized in Ba5K25. The only sperm fusion in this egg was accompanied by the response marked "a". The time of fusion being indicated by the arrow above the picture. "b" denotes the small depolarizations recorded about 1 minute after sperm fusion. B shows the depolarization elicited by a sperm fusion in an egg bathed in Ba10K25. The current clamp potential in this egg was -93mV.


A
Sp 1
a
b
-40
-60
-80
-100
Current clamp potential (mV)
1.0
2.0
3.0
B
Sp 1
-40
-60
-80
-100
1.6
2.0
Time (mins)

In one of these eggs fertilized in Ba5K25, the sperm fusion was in synchrony with the electrical event marked "a" in Fig.6.1A. About one minute after sperm fusion a couple of small depolarizations were noted (marked "b" in Fig.6.1A), but these were not elicited by a further sperm fusion. This monospermic egg appeared very granular during histological examination. This may be explained by the absence of calcium. The depolarization "a" in Fig.6.1A was slightly reminiscent of those illustrated in Chapter 5. There is a fast upstroke phase (like an action potential) superimposed on a depolarization. The current clamp potential was -94mV, the duration of the whole of the depolarization marked "a" was 56 seconds and its peak was -48mV. In a further 12.2 minutes of recording after the event marked "b" no electrical event was noted.

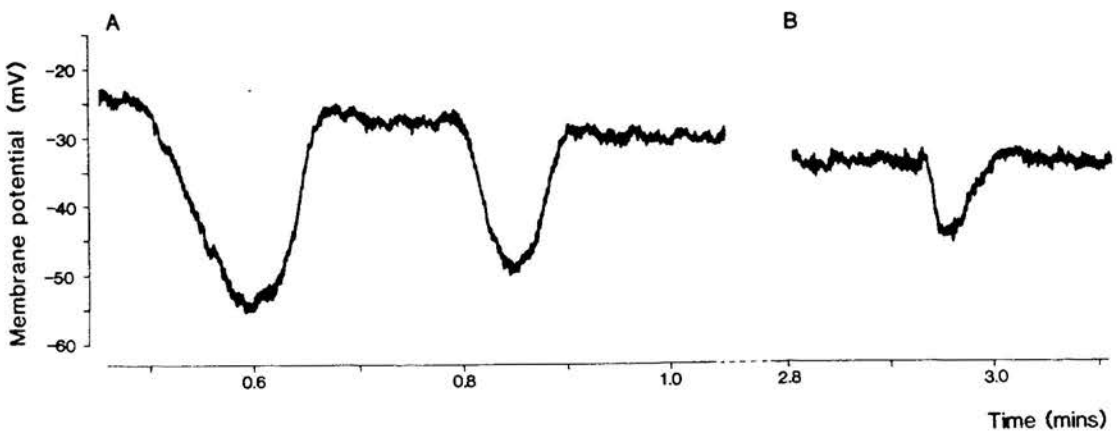
In another egg, a small depolarizing event accompanied the sperm fusion, shown in Fig.6.1B. This egg bathed in Ba10K25 was later confirmed by histology to be monospermic.

6.2 Fertilizations in calcium-free, lanthanum substituted solutions

Of the two eggs fertilized in La1K5 (i.e. calcium-free normal solution containing 1mM lanthanum), in only one was an electrical response recorded (Fig.6.2). This egg was unclamped when fertilized. Its membrane potential and input resistance was -33mV and 240MΩ respectively. In this egg three trhs were recorded

Figure 6.2

A. The first two trhs recorded in an egg fertilized in LalK5, occurred at 0.5 and 0.8 minutes respectively. The third trh recorded in the same egg, shown in B occurred at 2.9 minutes.


A
B
Membrane potential (mV)
-20
-30
-40
-50
-60
0.6
0.8
1.0
2.8
3.0
Time (mins)

after fertilization (all of which are shown in Fig.6.2). The first trh was observed 29 seconds after insemination of the recording chamber. In a further 10.8 minutes of recording after the end of the third trh, no response was observed. The egg was confirmed by histology to be monospermic.

A hyperpolarizing shift was noticeable after the first trh (the membrane potential prior to the first and third trh was -23mV and -33mV respectively). The durations of each trh progressively decreased (10, 6 and 4 seconds, in order of occurrence) and the peak of each trh progressively depolarized (-54mV, -48mV and -42mV, in order of occurrence).

Unfortunately in this experiment it was not possible to be sure when the sperm became straight and immotile. This was because the sperm reached the egg and fused with it, very soon after addition to the chamber. No small depolarization was observed prior to the first trh.

6.3 Fertilizations in calcium-free, magnesium substituted solutions

In all, ten fertilization experiments were attempted in calcium-free magnesium substituted solutions. Three of them were in normal potassium (i.e. 5mM) and the remaining seven in a high potassium (i.e. 25mM) solution. The concentration of magnesium in all of them was 10mM. Only one egg was successfully

fertilized in Mg10K5, but this yielded no electrical event during recording. Of the four successful fertilizations in Mg10K25, in only three were electrical events noted. These are discussed below.

One egg was fertilized by two sperm in Mg10K25. The first sperm fusion was accompanied by an fsd (current clamp potential = -120mV, peak potential = -75mV, amplitude = 45mV, rate of depolarization = $34mVsec^{-1}$). Superimposed on the plateau of this fsd was a trd (amplitude = 22mV, duration = 8secs, peak potential = -56mV). The second fusion also evoked an fsd which had a permanent plateau (current clamp potential = -125mV, peak potential = -70mV, amplitude = 55mV and rate of depolarization = $40mVsec^{-1}$). Superimposed on this plateau was another trd (amplitude = 20mV, duration = 8secs, peak potential = -54mV and rate of depolarization = $8mVsec^{-1}$). Approximately 2.5 minutes later another trd was observed for which the reversal potential was calculated to be -50mV. The initial membrane potential and input resistance of this egg was -16mV and 130MΩ respectively. After fertilization the final membrane potential and input resistance was -21mV and 60MΩ, i.e. a hyperpolarization of 5mV and a decrease in the input resistance of 70MΩ occurred after fertilization.

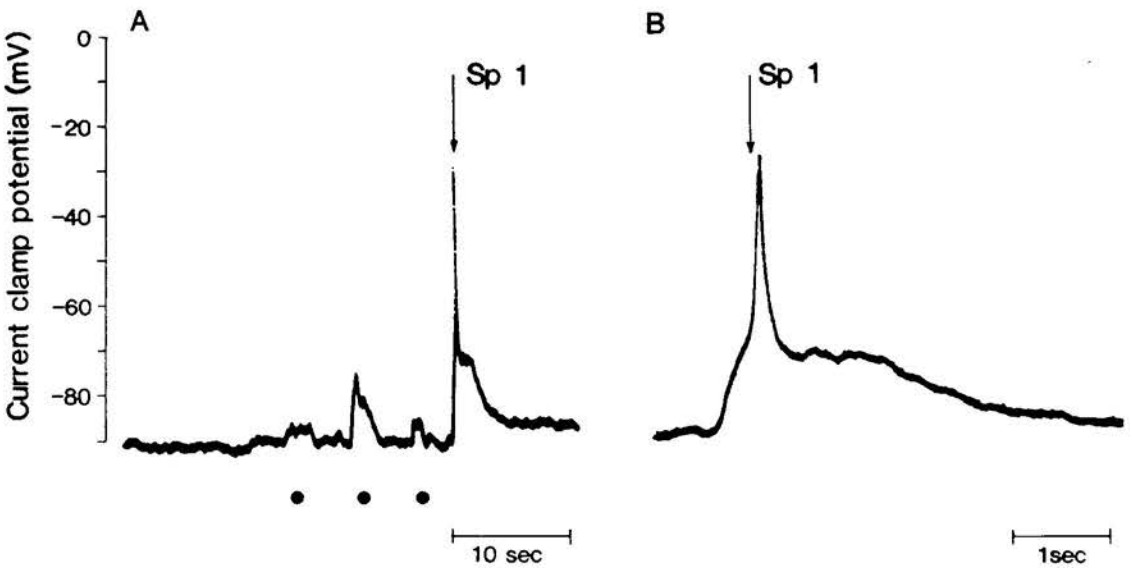
Another egg fertilized in Mg10K25, had a membrane potential of -14mV and an input resistance of 100MΩ. A trh was observed about 6.1 minutes after the addition of sperm to the chamber (the membrane potential just

prior to the response was -17mV and the reversal potential for the response was -46mV). A sperm was observed to straighten 10 seconds prior to the above mentioned trh - no depolarization was found to accompany this. In a further 6.2 minutes of recording after the trh, no further event was observed.The final membrane potential and input resistance of this egg was -11mV and 75MΩ. Histology showed this egg to be dispermic. The instant of fusion of the second sperm was not confirmed visually, because after the first fusion, many sperm attached to the egg, and then gradually decreased in motility.

In a third egg fertilized in Mg10K25, a depolarization (fsd) was observed to accompany the first sperm fusion. This egg had been current clamped to a potential of -83mV prior to adding the sperm to the recording chamber. No further electrical events were noted, even though four further sperm subsequently fused with the egg. During the plateau of the depolarization caused by the first fusion the most positive potential was -66mV, hence had there been any electrical events due to changes in the calcium activated potassium conductance, then theoretically they should have been observed (in the form of trds). But instead sperm fusions appeared to be accompanied by very small "bursts of noise" (small oscillations of potential, the amplitude of which were less than 5mV), which lasted as long as 40 seconds. In total, the egg

Figure 6.3

A Oscilloscope picture of a sperm evoked action potential recorded in Sr10K5 (marked by an arrow above the trace). Three small depolarizations were recorded before the action potential (marked by filled circles before the trace), which have been interpreted as being events linked to sperm fusion. B is an oscilloscope picture showing the action potential in A on an expanded time base. The current clamp potential of this egg was -90mV.


A
B
Current clamp potential (mV)
0
-20
-40
-60
-80
Sp 1
Sp 1
10 sec
1sec

was fertilized by five sperm.

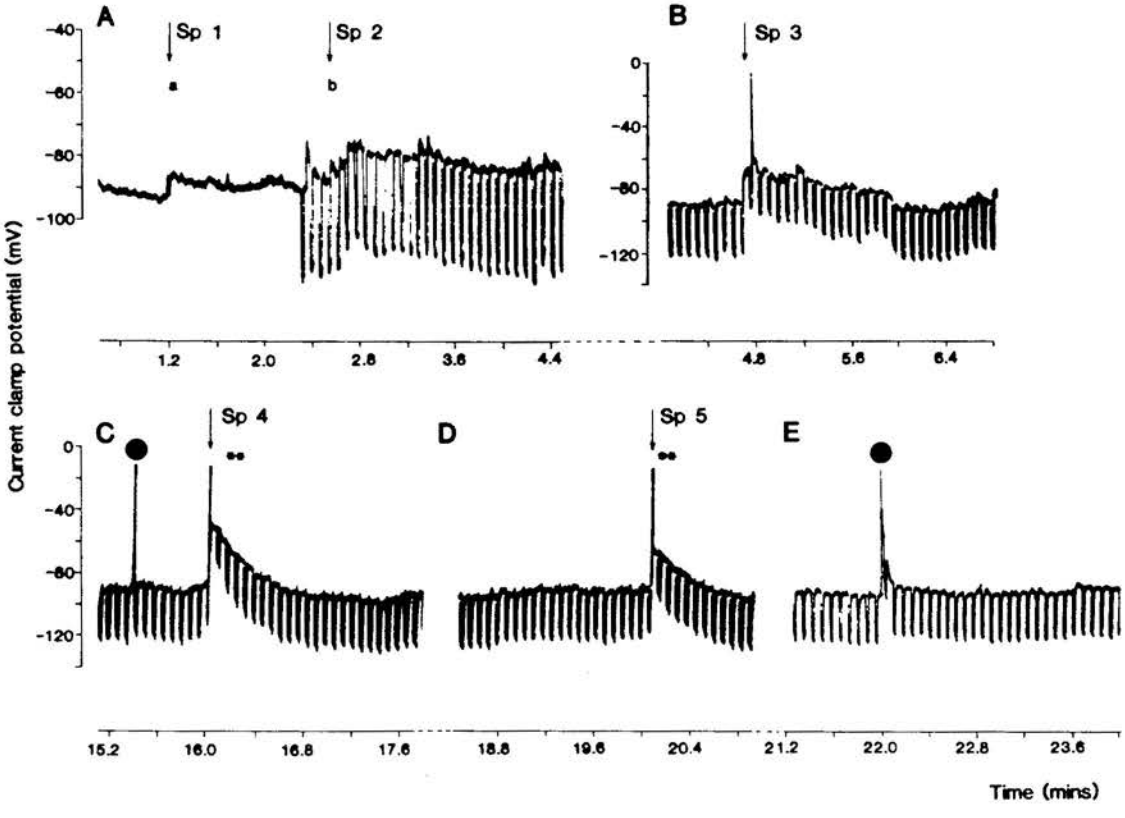
6.4 Fertilizations in calcium-free strontium substituted, normal potassium solutions

A monospermic fertilization in Sr10K5, gave rise to a sperm evoked action potential at the instant of sperm fusion (Fig.6.3A and 6.3B). In Figure 6.3B the action potential shown in Fig.6.3A is shown at a faster sweep speed. Just before this action potential, small depolarizing events were observed which were associated with sperm fusion (marked by filled circles in Fig.6.3A). The current clamp potential was -90mV, the peak of the action potential was -26mV, its duration was about 4 secs, and the plateau potential level was about -71mV. The threshold of the sperm evoked action potential was more hyperpolarized than that of the electrical evoked action potential in this egg (-66 and -58mV respectively). No other electrical events were recorded. The membrane potential before fertilization and after fertilization was -36mV and -40mV respectively, (i.e. a hyperpolarization of 4mV was observed in this egg after fertilization). The rate of depolarization (of the "event" which takes the membrane potential to the threshold of the sperm evoked action potential) was $62mVsec^{-1}$.

The oscilloscope pictures shown in Fig.6.4 are the electrical events observed during fertilization of another egg in Sr10K5. On impalement this egg had a membrane potential and an input resistance of -32mV and

Figure 6.4

Oscilloscope pictures of the responses observed during fertilization of a zona-free hamster egg in Sr10K5. The egg was current clamped at -95mV prior to insemination. the arrows above each trace indicate the timing of each fusion, (A-D). Letters "a" and "b" refer to the fsds elicited by the first and second fusion respectively. Asterisks above the trace (in C and D) refer to the presence of anode break spikes which have been blanked off for clarity. Filled circles above the traces denote brief action potentials (C and E). At 2.2 minutes hyperpolarizing pulses were passed through the electrode to monitor changes in conductance (0.15nA, 1 second, 0.22Hz). Note that the "potential gain" in B-E is half that in A.


A
Sp 1
Sp 2
a
b
B
Sp 3
C
Sp 4
D
Sp 5
E
Current clamp potential (mV)
Time (mins)

240MΩ respectively. It was then current clamped to -95mV prior to insemination. The first three sperm fusions were accompanied by the electrical events shown in Aa, Ab and B respectively. Depolarizations (fsds) accompanied the first two sperm fusions (Aa and Ab) whereas an action potential was correlated with the third (B). After the first sperm fusion, hyperpolarizing current pulses were passed through the electrode to allow simultaneous conductance measurements (at 2.2 minutes). The third fusion was followed 7.1 minutes later (at 11.8 minutes) by a trh which is not illustrated. The reversal potential of this trh was -80mV and the current clamp potential at it's onset was -74mV. A fourth and fifth fusion (each eliciting an action potential) occurred at 16.0 and 20.1 minutes respectively (as shown in Fig.6.4C and 6.4D respectively). Due to the hyperpolarizing pulses being used to monitor the conductance anode break spikes were observed just after the fourth and fifth fusions. Each of these has been blanked off for clarity, but their occurrence has been denoted by asterisks in Fig.6.4C and 6.4D. Thirty-seven seconds prior to the action potential elicited by the fourth fusion a brief action potential was recorded (see 5.24). This is shown in Fig.6.4C and is marked by a filled circle above the trace. Similarily another brief action potential was recorded about 22.0 minutes after insemination of the recording chamber shown in

Figure 6.5

Recordings from an egg fertilized in Sr5K25. A is a pen trace recording showing some of the responses observed during this experiment. Letters c,d and f refer to the corresponding oscilloscope pictures of the responses shown in B. The asterisk refers to a small depolarization observed about 30 seconds prior to the sperm evoked action potential. Ba is an oscilloscope picture of the depolarization which accompanied the first fusion (marked by an arrow above the trace). b and c are two trd spikes which followed the first fusion. d and e are pictures of the sperm evoked action potential caused by the second fusion (marked by arrow above both traces). The asterisk in d has the same meaning as in A above, f shows a burst of noise which followed the second fusion. The egg was initially current clamped at -73mV, then after the first fusion the constant current passed through the electrode was increased to current clamp the egg at -100mV.

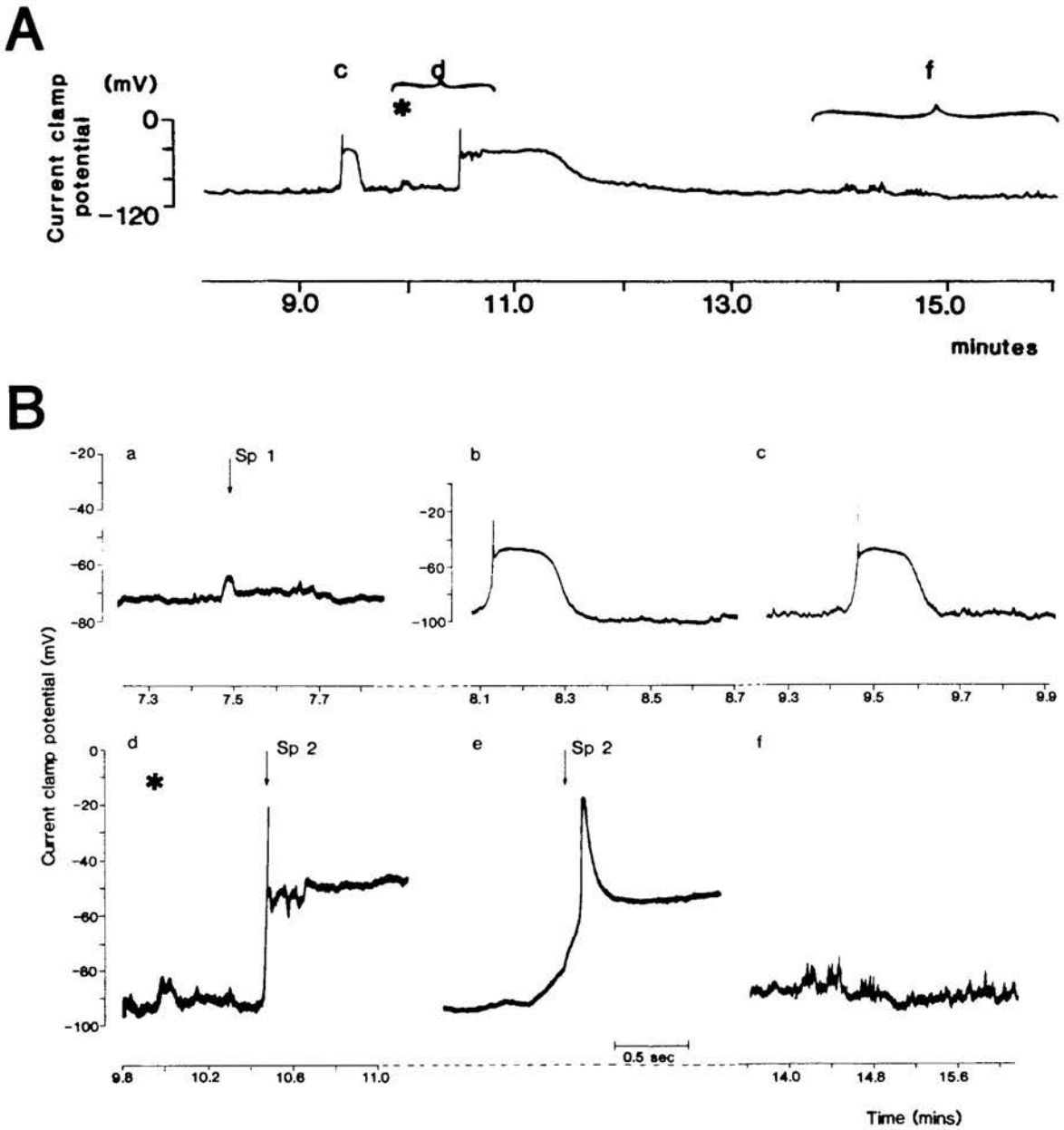

A
Current clamp potential
(mV)
0
−120
c
d
f
*
9.0
11.0
13.0
15.0
minutes
B
Current clamp potential (mV)
a
Sp 1
b
c
d
Sp 2
e
Sp 2
f
*
0.5 sec
Time (mins)

Fig.6.4E). Neither of these were associated with sperm fusions. The reversal potential of the fsd accompanying the second fusion was +24mV (rate of depolarization <1mV sec^{-1}). The reversal potentials of the plateaus of the action potentials which accompanied the third, fourth and fifth fusions were +31, +20 and +106mV respectively.

6.5 Fertilizations in calcium-free, strontium substituted, high potassium solutions

An example of a recording from an egg fertilized in Sr5K25 is shown in Fig.6.5. The first sperm fusion elicited a fsd (duration = 16 seconds, amplitude = 10mV, peak = -63mV), at about 7.5 minutes after insemination of the recording chamber (Fig.6.5Ba). This was followed by two trd spikes at 8.1 minutes (Fig.6.5Bb) and at 9.4 minutes (Fig.6.5A and Fig.6.5Bc). The second sperm fusion occurred at 10.4 minutes (Fig.6.5A, 6.5Bd and 6.5Be). This elicited a sperm evoked action potential, which was quite different in duration to the two preceding trd spikes. The duration of the action potential was 111 seconds (that of the two trd spikes was 17 and 14 seconds). But interestingly the peak (not the spike peak) of the two trd spike responses (-47mV and -50mV) was similar to the plateau potential level of the sperm evoked action potential (-49mV). It may be that the calcium activated potassium conductance which plays a major role in the trd spike response, may also play a

predominant part in the plateau of a sperm evoked action potential. About 2.1 minutes after the end of the action potential a burst of "noise" was recorded which presumably is due to some event triggered by the sperm fusion (Fig.6.5A and 6.5Bf) (occurring between 14 and 15 minutes after insemination).

The threshold of the sperm evoked action potential was -62mV and its spike peak was -18mV (the spike peak of the two trd spikes illustrated in Fig.6.5Bb and Fig.6.5Bc was -27mV and -16mV, respectively. The threshold and the spike peak of the electrically evoked action potential in this egg was -62mV and -20mV respectively.

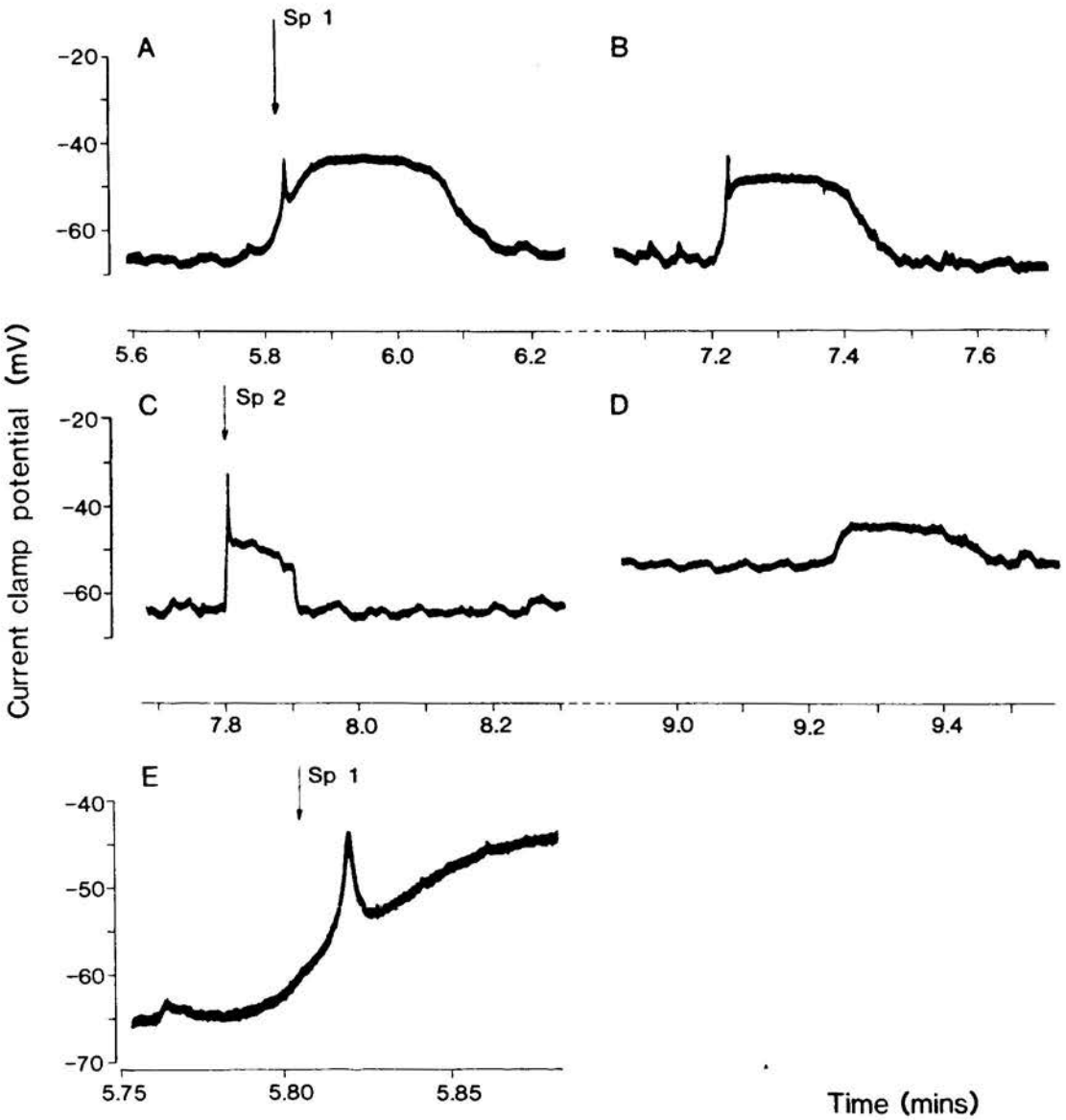
Just prior to the sperm evoked action potential shown in Fig.6.5Bd there was a small depolarization marked by an asterisk in 6.5A and 6.5Bd). This may be related to the fusion of the second sperm. Histology showed that this egg was fertilized by two sperm.

It is curious, that the first sperm fusion was associated with a very small depolarization whereas the second elicited an action potential which was quite different from the trd spike.

Figure 6.6 shows the responses recorded in an egg fertilized by two sperm in Sr5K25. The first fusion evoked a trd spike response (Fig.6.6A, also shown on a faster time base in Fig.6.6E), which was followed by another of shorter duration at 7.2 minutes (Fig.6.6B). The second fusion then elicited a sperm evoked action

Figure 6.6

Oscilloscope pictures of the first four responses recorded in an egg fertilized in Sr5K25. The initial current clamp potential was -67mV. The arrows above A and C indicate the timing of the two fusions in this egg. E. Rising phase of the response shown in A, at a faster time base.


Sp 1
A
B
-20
-40
-60
5.6
5.8
6.0
6.2
7.2
7.4
7.6
C
Sp 2
D
-20
-40
-60
7.8
8.0
8.2
9.0
9.2
9.4
E
Sp 1
-40
-50
-60
-70
5.75
5.80
5.85
Current clamp potential (mV)
Time (mins)

potential shown in Fig.6.6C, which was followed by a trd response at about 9.2 minutes (Fig.6.6D). The peak potential of the responses shown in A-D was -44, -48, -47 and -45mV respectively and the durations were 23, 16, 6 and 14 seconds respectively.

It is interesting that when the first sperm fused with this egg, no fsd or sperm evoked action potential was observed. But a trd spike was noted (Fig.6.6A), the duration of which was significantly longer than the other two slow responses observed (Fig.6.6B and 6.6D). The durations of both the slow responses , i.e. the trd spike (B) and the trd (D) was similar, being 16 and 14 seconds respectively.

After the response shown in D the current clamp was reduced, such that the current clamp potential was -48mV (current clamp potential prior to insemination was -67mV). At this potential two trds were observed the reversal potentials of which were -40 and -36mV (not shown in Fig.6.6).

6.6 Membrane potentials and input resistances of eggs in various calcium-free solutions, containing substituted multivalent ions (Table 6.1)

The membrane potentials ($p < 0.01$: Fisher Behrens test) and input resistances ($p < 0.01$: Fisher Behrens test) increased with increasing strontium concentrations. In Sr5K25, the membrane potential was -29 ± 5mV, n=20 and the input resistance was $160 \pm 60 M\Omega$, n=20, whereas in Sr10K5 the membrane potential was

Table 6.1

This summarizes the number of fertilization experiments performed in the various calcium-free solutions, with substituted multivalent ions, and the number of these eggs which were successfully fertilized. The membrane potentials and input resistances are also shown in the form mean $\pm$ SD, (n=), but when the number of results was too small the actual values have been listed (in italics). The Table also summarizes the changes in potential and resistance observed after fertilization. A negative value denotes a hyperpolarization or a decrease in resistance respectively.

SOLUTION	Number of successful fert^{n} / No of intracellular recordings	Membrane potential mean ± SD (n=) (mV)	Input resistance mean ± SD (n=) (MΩ)	Difference between the membrane potential before & after fert^{n} (mV) mean ± SD (n=)	Difference between the input resistance before & after fert^{n} (MΩ) mean ± SD (n=)
Ba2K25	0/2	-29 -41	160 220		
Ba5K25	5/8	-36±8 (8)	210±60 (8)	+5 +17 +17 +24	
Ba10K25	1/1	-45 -61	340 410	+20	
La1K5	2/5	-42±7 (8)	240±30 (8)	-3 +18	
Mg10K5	1/3	-21 -25 -35	80 90 130		
Mg10K25	4/7	-19±5 (6)	110±20 (6)	+3	-25
Sr10K5	6/8	-40±8 (8)	250±40 (8)	-5 -4 +2	-100
Sr5K25	12/19	-29±5 (20)	160±60 (20)		

TABLE 6.1

-40±8mV, n=8 and the input resistance was 250±40 MΩ, n=8. Although the higher membrane potential and input resistance in the latter may be as a consequence of the lower potassium concentration.

The input resistance of eggs bathed in Ba5K25 was not significantly different to that in Sr5K25 ($p>0.1$: two sample t test) but the membrane potential was higher in Ba5K25 compared to that in Sr5K25 ($p<0.05$: two sample t test).

The membrane potentials and input resistances in La1K5 (-42±7mV, n=8 and 240±30MΩ, n=8 respectively) were not significantly different to those in Sr10K5 or Ba5K25.

The membrane potential and input resistance of eggs in Mg10K25 was less than that in all other solutions.

In 11 eggs the unclamped membrane potential was noted at the end of the record, after fertilization. In all but three eggs, a depolarization was observed after fertilization. This was also noted in the fertilizations performed in the various calcium solutions, discussed in Chapter 5.

One egg fertilized in Mg10K25 and another in Sr10K5 showed decreases in resistance after fertilization.

6.7 The thresholds and spike peaks of electrically and

Table 6.2

This summarizes the thresholds and spike peaks of both the electrically evoked and sperm evoked action potentials in Sr10K5 and Sr5K25. Results are presented as either mean ± SD (n=) or the actual values are listed (in italics) on occasions when only a few results were available.

SOLUTION	ELECTRICALLY EVOKED ACTION POTENTIAL		SPERM EVOKED ACTION POTENTIAL	
	Threshold (mV)	Spike peak (mV)	Threshold (mV)	Spike peak (mV)
Sr10K5	-57±3(8)	-8±15 (8)	-66	-26
Sr5K25	-60±3(16)	-7±12 (15)	-56 -60 -74	-8 -10 -14 -22

TABLE 6.2

sperm evoked action potentials, in calcium-free solutions, containing substituted strontium (Table 6.2)

The threshold for electrically evoked action potentials was more negative in a lower divalent ion concentration ($p < 0.05$: two sample t test). The threshold in Sr10K5 was -57 ± 3mV, n=8 whereas that in Sr5K25 was -60 ± 3mV, n=16. There was no significant difference in the spike peak of electrically evoked action potentials in Sr10K5 and Sr5K25 (-8 ± 15mV, n=8 and -7 ± 12mV, n=15 respectively).

6.8 Measurements on the fast responses observed at fertilization in calcium-free solutions, containing substituted strontium (Table 6.3)

The limited number of experiments in this part of the study has meant that a complete statistical analysis was not possible.

The mean peak of the fsds was -62mV in Sr5K25, whereas the mean threshold of the electrically evoked action potential in the same solution was -60mV. These figures would indicate that fsds would not give rise to action potentials because they were subthreshold. But it should be noted that the results obtained in calcium containing solutions (Chapter 5) indicated that the mean threshold for sperm evoked action potentials was more hyperpolarized than that of the electrically evoked action potentials.

Further analysis of four eggs (fertilized in calcium-free strontium substituted solutions) which

Table 6.3

This summarizes the various parameters measured of the fast responses which were recorded in eggs fertilized in Sr10K5 or Sr5K25. The fast responses being either fsds or sperm evoked action potentials. Results are shown either as mean $\pm$ SD, (n=) or as the actual values (in italics) obtained.

SOLUTION	FSD				SPERM EVOKED ACTION POTENTIAL			REVERSAL POTENTIAL OF FSDs (mV)
	Duration (sec)	Peak (mV)	Amplitude (mV)	Rate of depol^{n} (mV sec^{-1})	Duration (sec)	Plateau potential level (mV)	Rate of depol^{n} (mV sec^{-1})	
Sr10K5	24 26 32	-62 -74 -85 -100	10 14 14 16	8	5 11 30 37 75	-71 -54±10 (6)	61 32 41 47 61	+38±49(6)
Sr5K25	28±30 (8)	-62±19 (9)	18±8 (9)	30±36 (9)				

TABLE 6.3

showed both fsds and sperm evoked action potentials, in which both the threshold for the electrically evoked and sperm evoked action potentials was known, indicated that the peaks of the fsds, in all four cases were more negative than the threshold for the sperm evoked action potentials. Also in all four cases the threshold of the sperm evoked action potential was more negative than that of the electrically evoked action potential.

The durations of the sperm evoked action potentials were variable (as was observed for fertilizations in calcium containing solutions) - the range in only four eggs being eleven to seventy-five seconds.

The plateau potential level of the sperm evoked action potentials in Sr5K25 was -54 ± 10mV, n=6, which is no different to that in Ca2K25 (-59 ± 8mV, n=5) ($p > 0.6$: two sample t test).

The rate of depolarizations of the sperm evoked action potentials in Sr5K25 were 32, 41, 47 and 61mVsec^{-1}.These are all larger than the mean rate of depolarization of the fsds in the same solution (30 ± 36mV sec^{-1}, n=9).

The reversal potential of the fsds in Sr10K5 was found to be $+38\pm49$mV, n=6.

6.9 Measurements on the slow responses observed during fertilization in calcium-free solutions, containing substituted strontium (Table 6.4)

The durations of trds in three eggs bathed in Sr5K25 were 14, 19 and 19 seconds whereas that of the trd spikes was 16±3 seconds, n=8. The peaks of the trds in the three eggs were -45, -45 and -49mV whereas that of the trd spikes was -48±2mV, n=9. Thus it appears that the peaks and durations of trds and trd spikes are not significantly different from each other.

The spike peak of the trd spike (-21±13mV, n=9) was more hyperpolarized than that of the electrically evoked action potential (-7±12mV, n=15) in Sr5K25 ($p<0.02$: two sample t test).

Three reversal potential measurements were made in Sr5K25 of trds: -36, -40 and -42mV.

6.10 Discussion

Many of the types of responses discussed in Chapter 5 were found to be observed in fertilizations in calcium free solutions also:-

a) sperm fusion in eggs current clamped at high potentials, in barium substituted solutions was accompanied by a depolarization (Fig.6.1).

b) fertilization of a low membrane potential egg bathed in lanthanum substituted solutions, elicited trhs (Fig.6.2) which were superimposed on a hyperpolarizing shift. The amplitude and duration of each successive

Table 6.4

A summary of the various parameters measured of slow responses recorded in eggs fertilized in Sr5K25. The slow responses were trds and trd spike (no trhs were recorded). Results are shown as either mean ± SD, (n=) or the actual values (in italics) are quoted.

SOLUTION	trd				trd - spike			
	Duration (sec)	Peak (mV)	Rate of depol^{n} (mV sec^{-1})	Reversal potential (mV)	Duration (sec)	Peak (mV)	Rate of depol^{n} (mV sec^{-1})	Spike peak (mV)
Sr5K25	14 19 19	-45 -45 -49	9 10	-36 -40 -42	16±3 (8)	-48±2 (9)	5 8 26 30	-21±13 (9)

TABLE 6.4

trh decreased. Such differences between successive trhs were also noted during fertilizations in calcium containing solutions (Figures 5.1 and 5.2).

c) fsds, trds and permanent plateaux were observed during fertilization of eggs current clamped at high potentials and bathed in magnesium substituted, calcium free solutions. A trh was also noted during the fertilization of another egg, not current clamped, but bathed in a similar solution.

d) sperm evoked action potentials and fsds were recorded during fertilizations in strontium substituted normal potassium solutions. As in calcium containing solutions (5.22) the threshold of a sperm evoked action potential was found to be more negative than that of the electrically evoked action potential.

e) during a fertilization in strontium substituted, high potassium solution, trd spikes, an fsd and a sperm evoked action potential were observed (the threshold of which was no different to that of the electrically evoked action potential). In another similar fertilization the trd spike accompanying a sperm fusion was longer in duration than the others recorded in the same egg- as discussed in 5.15 (see Fig.5.21).

Although similar electrical responses are recorded in calcium free, multivalent ion substituted solutions the success rate of fertilization was lower in those solutions containing barium, lanthanum and magnesium. This may be explained by a detrimental effect of the absence of calcium on the sperm and/or the egg. An

indication of this may be the lower membrane potentials and input resistances observed in magnesium substituted solutions.

Yanagimachi (1978a) reported that 0.2mM calcium in the extracellular medium was sufficient to allow 100% penetration of eggs. Whereas concentrations of 2-5mM barium, strontium and magnesium were required for similar penetration rates. In the same study the author also reports that the sperm swam much less actively in calcium-free media.

In the present study, the effect of calcium-free media on parthenogenetic activation was not investigated. Although Whittingham and Siracusa (1978) have reported that increasing concentrations of strontium and barium (up to 2.0mM) increased the rate of activation (of mouse oocytes removed 17 hours post-HCG) in calcium free media. Increasing magnesium did not increase the percentage activation (0.1 and 3.0mM).

Igusa and Miyazaki (1983) showed that the occurrence of trhs at fertilization requires the presence of external calcium. Furthermore such hyperpolarizing responses could be elicited by injections of calcium into the egg (Georgiou et al., 1983), and also by injections in calcium free media (Igusa & Miyazaki, 1983). These results suggest that a calcium influx is required for these responses.

Since fsds and sperm evoked action potentials were observed in calcium-free solutions substituted with

divalent ions (above) and also in low sodium solutions (5.7), then it is likely that these depolarizations accompanying sperm fusions are caused by an influx of ions through calcium channels. In the experiments described above, an influx of barium, magnesium or strontium through the calcium channels would be responsible for the sperm evoked depolarizations. These calcium channels may be those already present in the egg membrane (Okamoto et al., 1977; Georgiou et al., 1984; Chapter 4 of the present study), or those in the sperm membrane, which after fusion is incorporated into the egg. A depolarization will only be observed if there is an influx of cations (or an efflux of anions) across the "sperm-egg membrane combination". If the sperm head acts as a source of calcium after fertilization, then this will not be observed as an electr ical event (at the instant of fusion, although slow responses may nevertheless follow).

Basically there are three possible entry routes or sources of calcium in the sperm egg membrane combination:

a) calcium influx across the sperm membrane after it has fused with the egg membrane

b) calcium influx across the egg membrane through voltage sensitive calcium channels and

c) the sperm head acts as a source of calcium (Baker, 1980; Jaffe, 1980).

The fact that depolarizations (fsds) were observed in magnesium substituted calcium free solutions might

be because there are channels in the sperm membrane which allow entry of magnesium (since voltage sensitive calcium channels of the egg membrane are not permeable to magnesium; P. Georgiou, unpublished results). Another possibility is that the depolarization is caused by an efflux of anions (unfortunately no fertilization experiments were performed in the present study in chloride free solutions). Slow responses were recorded during fertilization experiments in magnesium substituted calcium free solutions. Such results therefore indicate that a release of calcium from cytosolic stores within the egg had been triggered off, by a calcium release from within the sperm head, since injections of magnesium into the egg do not evoke changes in the potassium conductance (Georgiou, 1985; Igusa and Miyazaki, 1983).

Similarly in lanthanum substituted calcium-free solution the fact that trhs were recorded indicates that they were initiated by a calcium release from a store within the sperm head, since lanthanum blocks the voltage sensitive calcium channels within the egg membrane (Georgiou et al., 1984) and presumably those in the sperm membrane.

Therefore from the experiments described in the present study, no conclusions can be drawn as to whether or not calcium is released from within the sperm head. Evidently there must be an ionic movement

across the sperm egg membrane combination at the time of fusion (due to the depolarization recorded). Furthermore, it could be argued that the calcium introduced into these "calcium free solutions", on addition of sperm (70μM maximum) may be sufficient to cause a depolarization in the magnesium or strontium substituted calcium free solutions.

In order to activate the eggs a very large, sudden increase in calcium within the egg is required, as indicated by the calcium injection experiments of Fulton and Whittingham (1978). A train of electrically evoked action potentials or the insertion of an intracellular microelectrode (which may cause a leak pathway and hence a calcium influx) does not cause parthenogenetic activation. The sperm induced cytosolic calcium increase, is different in one further respect, in that it is able to initiate a "train of trhs", whereas each calcium injection elicits only one such response. Also sperm evoked action potentials, have a different ionic basis to electrically evoked action potentials, because their thresholds, peak heights and durations are different.

Voltage clamp experiments on mouse oocytes indicate selectivity ratios amongst the alkali earth cations, Ca:Sr:Ba for calcium channels of 1.0:1.4:0.7 (Okamoto et al., 1977). Hence for a given number of calcium channels in the "sperm-egg membrane combination", this would mean that the current carried by strontium ions would be larger than that by calcium

ions (assuming that the concentration of both ions in the extracellular medium is the same). Similarly that carried by barium ions would be smaller than the current carried by calcium. Therefore in order to investigate the amplitudes of fsds and sperm evoked action potentials, it may be best to perform such experiments in strontium containing solutions. Although calcium may be "better" than strontium for some other step during the fertilization process.

CHAPTER 7 ELECTRICAL EVENTS, DURING HOMOLOGOUS FERTILIZATIONS OF ZONA-FREE HAMSTER EGGS RECORDED WITH EXTRACELLULAR PATCH PIPETTES

In Chapters 5 and 6 the electrical events accompanying homologous fertilizations of zona-free hamster eggs were monitored using intracellular electrodes. In this chapter similar experiments were performed, but the electrical recording was done with cell attached patch pipettes, a form of extracellular recording. The argument for doing these experiments was that, if a depolarization was associated with the initial contact between sperm and egg, it might manifest itself as an "action current".

It is stressed that convention was broken with regard to the extracellular currents displayed in this chapter. All inward currents are displayed as upward deflections (in this chapter only and Figure 6 of Chapter 4).

7.1 Methods

The patch pipettes had tip diameters and resistances similar to those used in the experiments described in Chapters 3 and 4. They were filled with one of two solutions. Either Ca10K5 (diluted to 90% with distilled water) or a solution which is designated as TEA-10 CsCl-120. The composition of the latter was mM: TEA (tetra-ethyl ammonium chloride) 10: Caesium chloride, 120; $MgCl_2$, 1.2; $CaCl_2$,10; Hepes,5 and NaOH, 2. 5. Patch pipettes were filled with TEA-10 CsCl-120 in an attempt to abolish potassium currents across the patch. The bathing solution in which the eggs were fertilized was one of the following:- Ca10K5

or Ca10K25 or Sr10K5.

The resistance between the patch pipette tip and the bath after application of mild suction was 0.2-4GΩ.

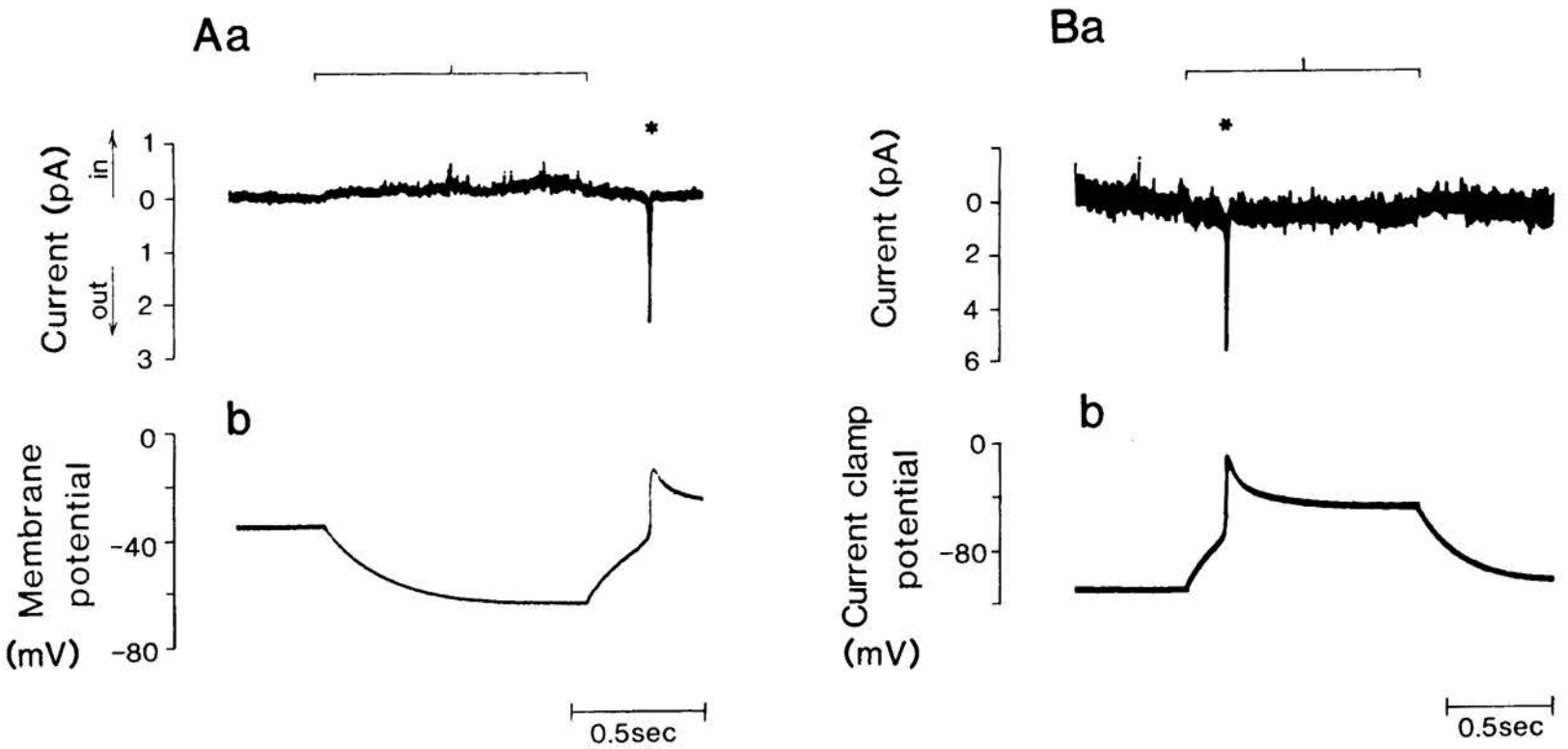
Intracellular recordings were made in a manner similar to that in experiments described in Chapters 5 and 6. Unfortunately it was not possible during all these experiments, to confirm the instant of fusion visually (indications of fusion times have been given in Figures, whenever it was possible).

7.2 Electrically evoked responses monitored by an intracellular electrode and an extracellular pipette simultaneously

In order to discover what extracellular current is measured by the patch pipette, during fast and slow depolarizations or hyperpolarizations, 16 eggs were impaled with an intracellular electrode after having obtained a cell-attached patch. It was then possible to pass hyperpolarizing or depolarizing current pulses through the intracellular electrode, or current clamp the egg at high potentials. Examples of such an experiment are illustrated in Figure 7.1. The responses illustrated in Figure 7.1A were obtained from an egg bathed in Ca10K5 (patch pipette filled with Ca10K5 diluted to 90%). The membrane potential of this egg was -35mV, and when a hyperpolarizing pulse was passed through the intracellular recording electrode, an anode break response was recorded by the

Figure 7.1

Oscilloscope pictures of the extracellular current recorded by a cell attached patch pipette at the same time as recording an anode break response (A) and an action potential (B) with an intracellular electrode. The membrane potential of the egg from which the response shown in A was recorded was -35mV and the anode break response was evoked by the passage of a hyperpolarizing pulse (0.15nA,1sec) through the intracellular electrode. The response shown in B was (from a different egg to that in A) was recorded in an egg current clamped at -108mV and the action potential elicited by the passage of a depolarizing pulse (0.2nA,1sec). Both eggs were bathed in Ca10K5 and the patch pipettes were filled with this solution diluted to 90%. The asterisks denote capacitative currents (see text) and the brackets refer to the presence of the inward current in Aa and the outward current in Ba. The length of the bracket indicates the duration of the hyperpolarizing pulse passed through the intracellular electrode in A, and the duration of the depolarizing pulse in B.


Aa
Current (pA)
in
out
1
0
1
2
3
*
b
Membrane potential
(mV)
0
-40
-80
0.5sec
Ba
Current (pA)
0
2
4
6
*
b
Current clamp potential
(mV)
0
-80
0.5sec

intracellular electrode (Figure 7.1Ab). The extracellular current recorded simultaneously by the patch pipette is shown in Fig.7.1Aa. A small inward current (about 0.3pA maximum, marked by the bracket above the trace in Aa) was observed in synchrony with the hyperpolarization shown in Ab (caused by the hyperpolarizing pulse). This inward current has been interpreted as being an ionic current flowing across the patch membrane, from the pipette into the egg. In synchrony with the spike of the anode break response, an outward current was recorded by the extracellular pipette (marked by an asterisk above the trace in Fig.7.1Aa). This has been interpreted as being an outward capacitative current across the patch membrane as a consequence of the inward calcium flux during the spike. There is an increase in noise in Fig.7.1A (during the hyperpolarization), which appears to be due to increased inward channel activity. This being indicative of the inactivation of these channels at more depolarized potentials.

Similarly in another egg (bathed in Ca10K5, patch pipette also filled with Ca10K5, diluted to 90%) current clamped at -108mV, an action potential was evoked by passing a depolarizing pulse through the intracellular electrode (shown in 7.1Bb). The corresponding extracellular current is illustrated in Fig.7.1Ba. The spike of the action potential was observed as an outward capacitative current by the extracellular pipette (indicated by the asterisk above

the trace in Fig.7.1Ba). A small outward current is also noted, the duration of which is longer than that of the depolarizing pulse (marked by bracket in Fig.7.1Ba) this being the outward ionic current flowing across the patch membrane, into the pipette as a consequence of the depolarizing current pulse.

The qualitative aspects of this interpretation are now discussed. When the egg is hyperpolarized either by passing steady hyperpolarizing current or passing a hyperpolarizing pulse through an intracellular electrode, then ionic current flows into the egg across the whole of the egg membrane including the patch. This is depicted schematically in Figure 7.2A.

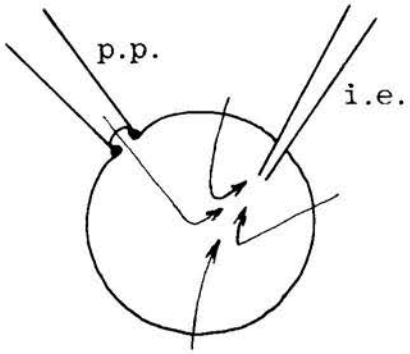
Similarly if the egg is depolarized either by injection of steady depolarizing current or a depolarizing pulse through an intracellular electrode, then ionic current will flow out of the whole of the egg membrane, including the patch (i.e. current will flow into the pipette; Figure 7.2B).

If inward currents flow across the egg membrane, such as during an anode break response (elicited by a hyperpolarizing pulse) or an action potential (elicited by a depolarizing pulse), then if the egg membrane and the patch membrane are isopotential for the entire duration of the anode break response or the action potential, inward channel currents should be observed in the patch (Figure 7.2D). In this situation no capacitative current should be observed across the

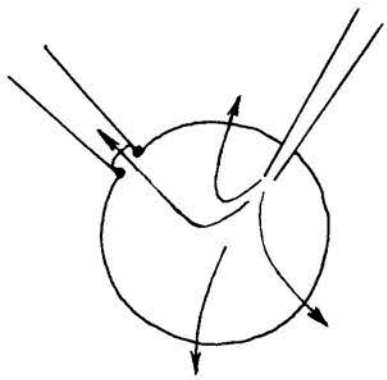
Figure 7.2

Schematic diagrams of currents marked by arrows, flowing across the egg membrane and the patch membrane. The patch pipette which functions as an extracellular pipette is marked P.P. in A (not marked in B-D). Enclosed by the internal diameter of the tip of the patch pipette is the cell attached patch. The intracellular electrode is marked i.e. in A (not marked in B-D). When the egg membrane is hyperpolarized by injection of hyperpolarizing current through the intracellular electrode, an inward ionic current is detected across the patch (A) and the rest of the egg membrane. When the egg membrane is depolarized by injection of depolarizing current through the intracellular electrode, an outward ionic current is detected across the patch (B), and the rest of the egg membrane. During anode break spikes and action potentials , inward ionic currents flow across the egg membrane (C and D), but if the egg membrane and the patch membrane are anisopotential an outward capacitative current will be observed across the patch (C). Such an outward capacitative current is not observed if the egg and the patch membrane are isopotential (D). Dotted line denotes the capacitative current and the solid lines depict ionic currents.

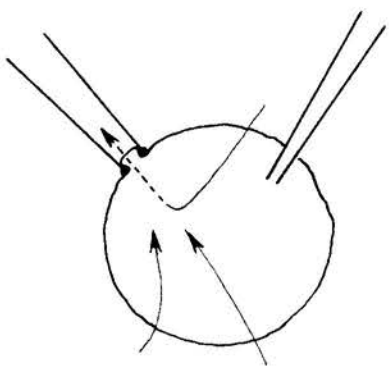
A HYPERPOLARIZATION



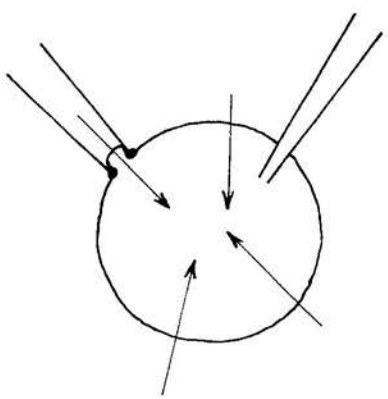
B DEPOLARIZATION



C ANISOPOTENTIAL



D ISOPOTENTIAL



patch. But as shown in Figure 7.1 an outward capacitative current was observed in coincidence with the anode break spike (Fig.7.1A) and the action potential (Fig.7.1B). As discussed in Chapter 4 inward channel currents were observed to accompany anode break spikes and action potentials (Figures 4.3 - 4.6). Therefore the egg membrane and the patch membrane must be anisopotential - this situation is depicted in Figure 7.2C.

In summary, an outward ionic current across the egg membrane due to a depolarization, is recorded as an outward ionic current across the patch by the extracellular pipette. Similarly hyperpolarizations of the egg membrane are recorded as inward ionic currents across the patch membrane by the extracellular pipette. All spikes, whether anode break or action potential, elicit outward capacitative currents across the patch.

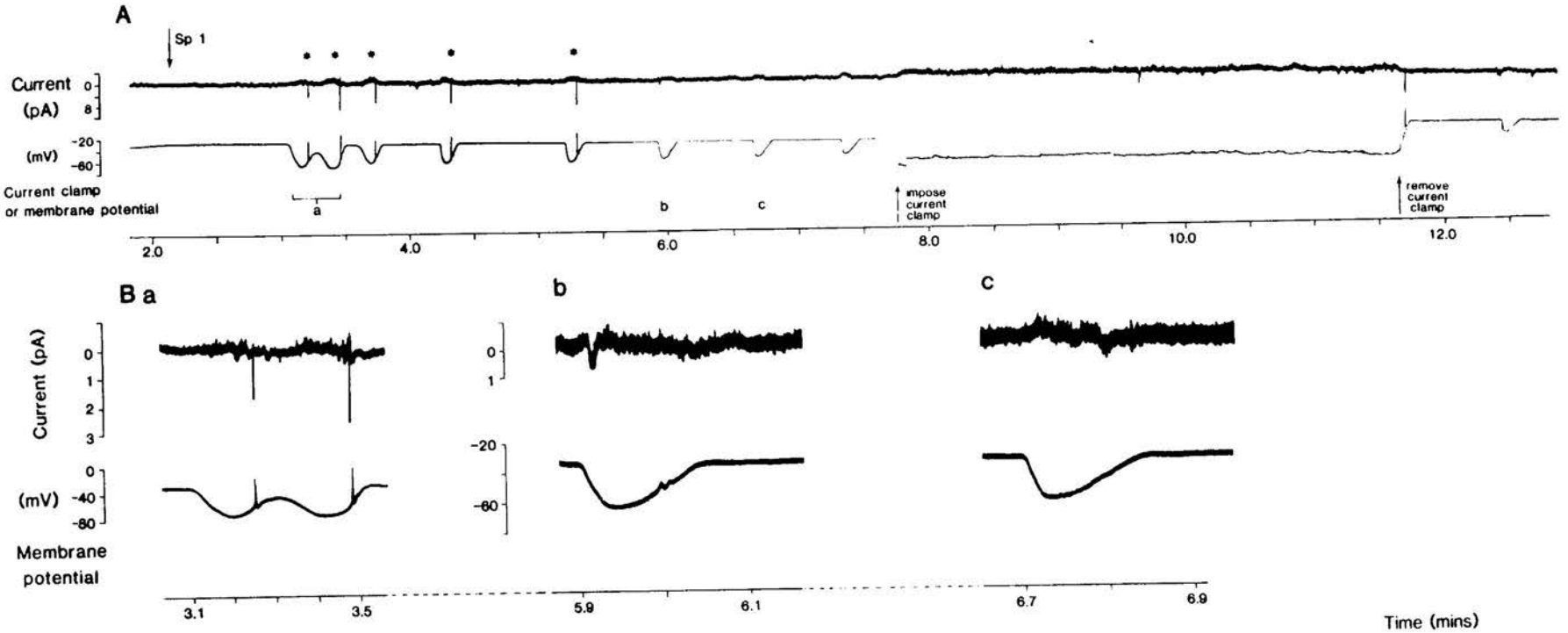
7.3 Fertilization responses recorded by an intracellular electrode and an extracellular pipette simultaneously

During the fertilization experiments described below the extracellular current was measured as well as the simultaneous membrane potential changes caused by sperm fusion. In all these experiments the egg was bathed in Cal0K5 and the patch pipette was filled with this solution diluted to 90%.

The responses from one such experiment are shown in Figure 7.3. The first fusion occurred at 2.2

Figure 7.3

A. Pen trace record of the extracellular current (upper) and the intracellular voltage (lower) measured in an egg fertilized in Cal0K5. The patch pipette was filled with Cal0K5 (diluted to 90%). The timing of the first fusion is shown by the arrow above the pen trace. Asterisks indicate outward capacitative currents measured by the extracellular patch pipette. Letters a-c below the intracellular record in A refer to the corresponding oscilloscope pictures in B. Arrows below the pen trace indicate the time at which current clamp was imposed and subsequently removed. Note: the steady inward current and the increase in noise recorded by the extracellular pipette, for the duration of the current clamp. Since the egg is hyperpolarized during the current clamp an inward ionic current is observed across the patch for the duration of the current clamp.


A
Sp 1
Current (pA)
0
8
(mV)
-20
-60
Current clamp or membrane potential
a
b
c
impose current clamp
remove current clamp
2.0
4.0
6.0
8.0
10.0
12.0
B a
b
c
Current (pA)
0
1
2
3
(mV)
0
-40
-80
-20
-60
Membrane potential
3.1
3.5
5.9
6.1
6.7
6.9
Time (mins)

minutes, when no extracellular current or voltage change is noted. At 3.1 minutes the first trh was recorded followed closely by another (both of which are illustrated in the oscilloscope picture shown in Fig.7.3Ba). But both these trhs had spikes superimposed on their respective repolarizing phases and therefore the trh and the spike, in combination have been called a trh spike response (in order to distinguish it from a trh not followed by a spike, this being called a trh response). A trh spike is recorded as a small inward current by the patch pipette, the duration of which approximated to that of the trh spike recorded by the intracellular electrode. This small inward current was followed by an outward current which was coincident with the spike of a trh spike response. The response recorded by the extracellular patch pipette at the same time as a trh spike response was recorded by the intracellular electrode, has been called a trh spike (I). Usually the trh spike (I) was only observed as an increase in noise on the extracellular current trace, as opposed to a distinct inward current.

The spike part of the trh spike is due to a calcium influx (Okamoto et al., 1977, Igusa & Miyazaki, 1983). This situation is analogous to anode break responses. The outward current recorded on the extracellular current trace at the same time as the spike of the trh spike is interpreted as being an

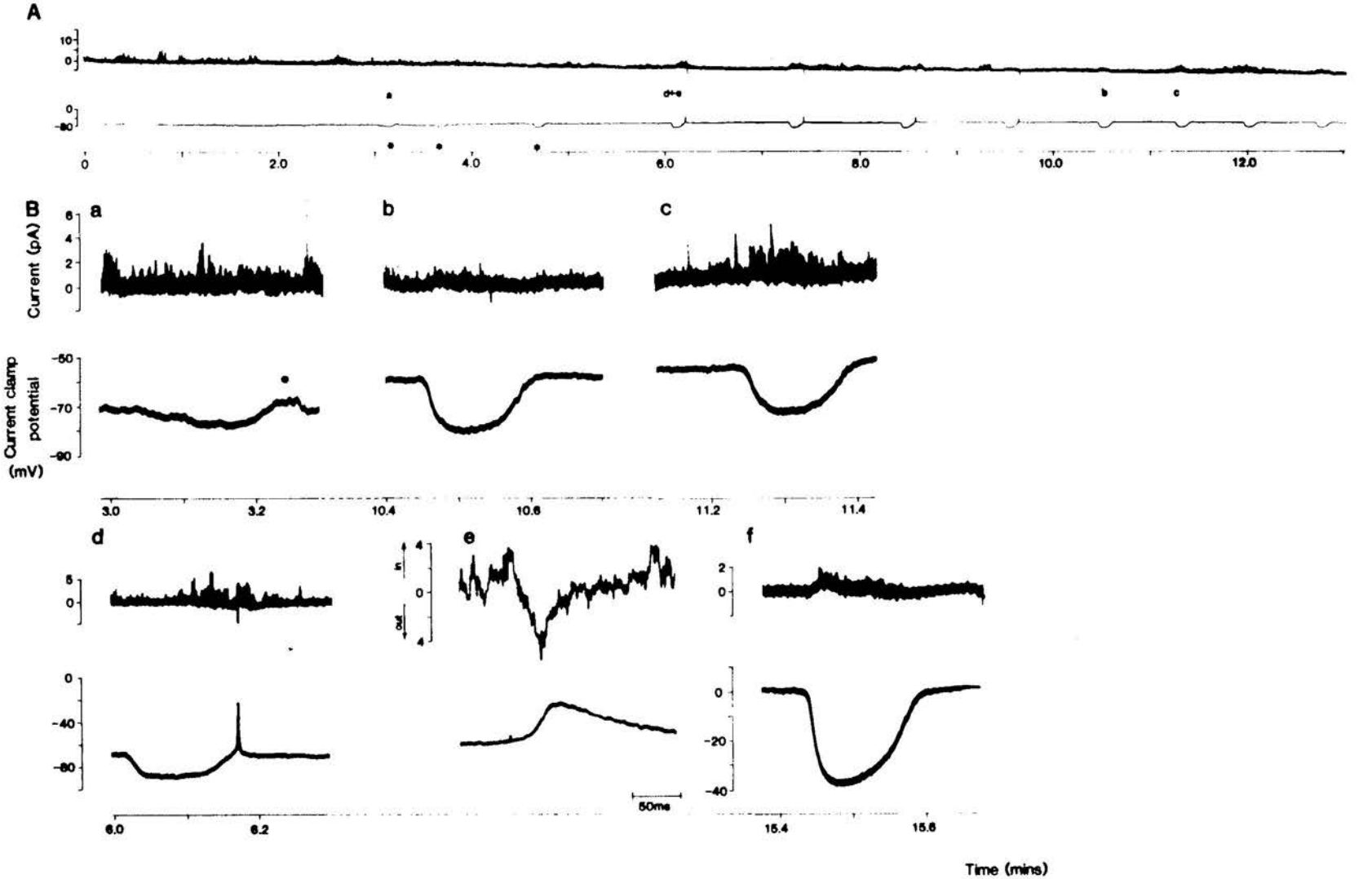
outward capacitative current due to the inward calcium flux, during the spike. These outward capacitative currents have been marked by asterisks above the extracellular current trace.

In all, five such trh spikes were recorded (the first five slow responses recorded in this egg). These were followed by three trhs, (Fig.7.3A) the first two of which occurring at 5.9 and 6.7 minutes are shown as oscilloscope traces in Bb and Bc respectively. The trhs were recorded on the extracellular current trace as small inward currents, called trh(I)s.

At 7.7 minutes the egg was current clamped at -74mV. In a further 4.0 minutes, whilst current clamped at this potential, no response could be clearly distinguished. When the current clamp was removed at 11.7 minutes the "train of trhs" and the coincident "train of trh(I)s" resumed. The first of these is shown on the pen trace in Fig.7.3A at 12.4 minutes. From these results, it was apparent that the trh(I)s recorded by the extracellular electrode, were very small (eg those shown in A had amplitudes of 0.4,0.4,1.2 and 0.4pA, in order of occurrence), whereas the coincident trhs recorded by the intracellular electrode had amplitudes of 29, 28, 25 and 21 mV in order of occurrence, i.e. in experiments in which the responses were recorded by just extracellular pipettes it was quite possible that trh(I)s might be incorrectly interpreted, or even missed. All measurements of amplitudes of extracellular currents were made from

Figure 7.4

A. Pen trace record of the extracellular current (upper) and the simultaneous intracellular potential (lower) recorded during the fertilization of an egg bathed in Ca10K5 (same solution diluted to 90%, was used to fill the patch pipette). Letters a-e refer to the corresponding oscilloscope pictures in B. Asterisks indicate the first three trhs recorded. In all the oscilloscope traces shown in B, the upper trace is the extracellular current record and the lower trace is a simultaneous intracellular potential record. A filled circle in Ba is to draw attention to the after-depolarization accompanying the first trh.


A
10
0
0
-80
a
d+e
b
c
0
2.0
4.0
6.0
8.0
10.0
12.0
B
a
b
c
Current (pA)
6
4
2
0
Current clamp potential (mV)
-50
-70
-90
3.0
3.2
10.4
10.6
11.2
11.4
d
e
f
5
0
4
0
4
0
-40
-80
2
0
0
-20
-40
50ms
6.0
6.2
15.4
15.6
Time (mins)

oscilloscope traces, and not pen records.

Similarly trh spike(I)s had small amplitudes (the five shown in A had amplitudes of 0.1,0.2,0.4,0.3 and 0.2pA in order of occurrence), when recorded by the extracellular pipette whereas the coincident trh spikes recorded by the intracellular electrode had amplitudes (the amplitude of a trh spike is the difference in the current clamp or membrane potential just prior to the response and the most hyperpolarized potential reached by such a response) of 40,40,30,33 and 34mV respectively.

The experiment discussed above, was one in which the egg was fertilized at a low membrane potential. The three examples below are ones in which the eggs were current clamped at high potentials before insemination. The results from one such experiment are illustrated in Figure 7.4. This egg fertilized in Ca10K5 (patch pipette filled with Ca10K5 diluted to 90%) was current clamped at -75mV prior to insemination. The first response recorded in this egg was at 3.0 minutes. It was a trh (Fig.7.4A and 7.4Ba) followed by two others at 3.6 and 4.6 minutes (all three being marked with asterisks below the intracellular record in Fig.7.4A). It was not possible to discriminate the presence of the trh on the extracellular current record (eg Fig.7.4A and Fig.7.4Ba). Close examination of the three trhs, reveals the presence of a small after depolarization

(on all three trhs) - more easily seen on the oscilloscope picture Fig.7.4Ba (marked by filled circle in Fig.7.4Ba).

At 6.0, 7.2, 8.4 and 9.5 minutes trh spikes were recorded on the intracellular record. The first of these is shown in Fig.7.4Bd, which is also shown on a faster sweep speed in Fig.7.4Be. The amplitudes of these trh spikes (18,22,24 and 24mV in order of occurrence) was larger than the previously observed trhs (amplitudes of which were 7,6 and 12mV in order of occurrence). Successive slow responses were getting larger, as expected, since all of them were superimposed on a depolarizing shift of the current clamp potential. Hence the current clamp potential prior to each response was further and further away from the reversal potential of these slow responses -see Chapter 5.

Further recording revealed the presence of trh responses, four of which are shown in A, at 10.5,11.3, 12.0 and 12.7 minutes. The first two of these are shown as oscilloscope traces in Bb and Bc respectively. It was not possible to distinguish an inward current across the patch membrane at the same time as these trhs.

A couple of important conclusions are drawn from such results. Firstly that when the current clamp potential is near -80mV, then a trh is not easily discriminated on the extracellular current trace nor on the potential record because the current clamp

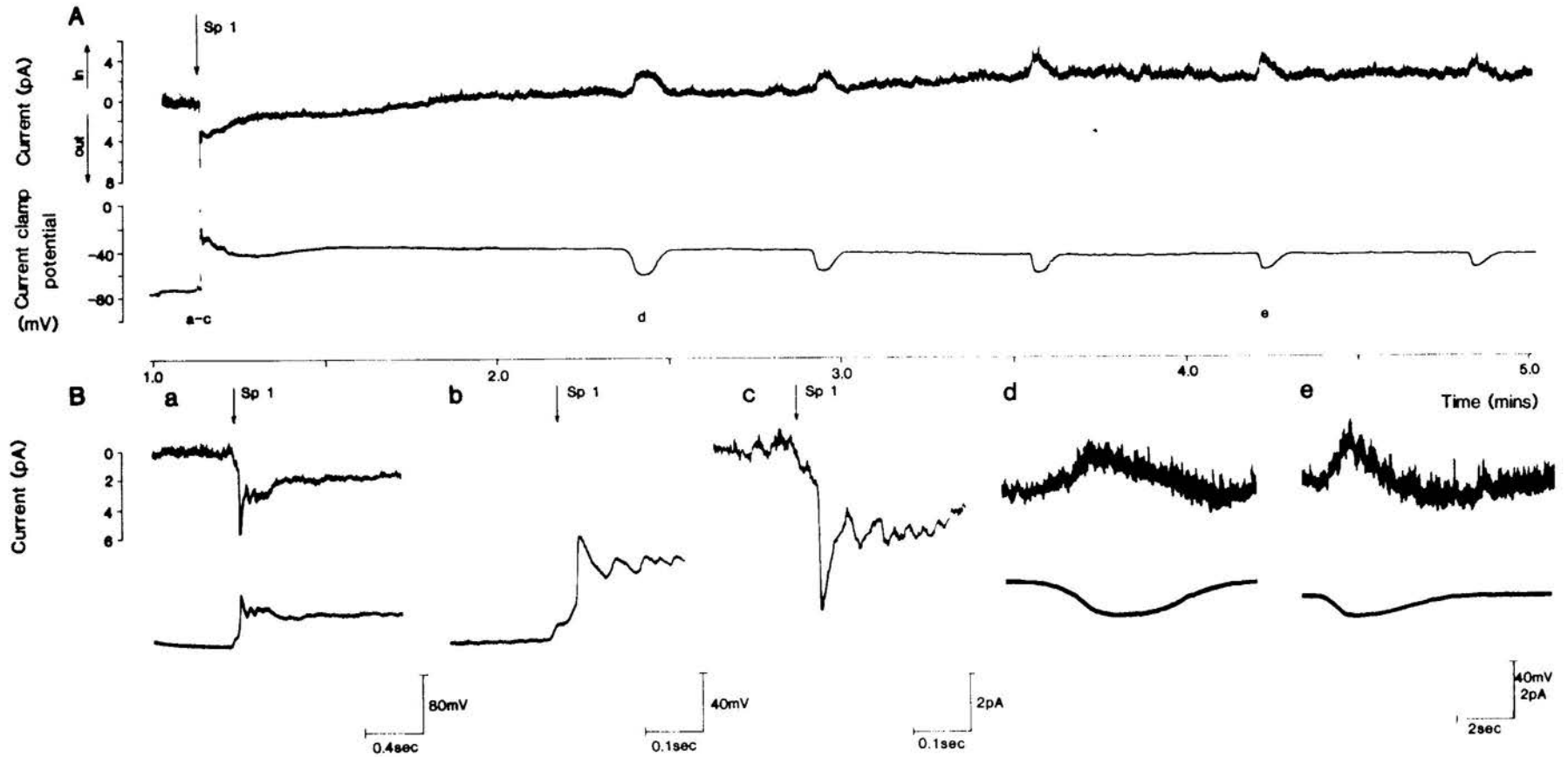
potential is approximately equal to the reversal potential of these slow responses (Chapter 5). Secondly if the slow responses occur in the order trhs, trh spikes and then trhs then this is compatible with a gradual depolarization or hyperpolarization of the membrane potential. A gradual depolarization occurred in the example illustrated by Figure 7.4. This is because when the current clamp potential was more negative than the threshold of the anode break response, then trhs were recorded. After further depolarization of the current clamp potential, trh spikes were recorded because the repolarizing phase of the trh "passed through" the threshold of the anode break response. Subsequently trhs were recorded, even though the peak of the trh was more negative than the threshold of the anode break response (presumably because the hyperpolarization during the trh is insufficient to overcome the inactivation of the calcium channel, the opening of which is responsible for the anode break response).

In all the fertilization experiments monitored with just intracellular electrodes only on one occasion was a trh spike recorded and that was in the group of experiments described in Appendix A.

At 15.2 minutes (not shown in Fig.7.4A) the egg was current clamped at 0mV, at which potential a trh was recorded, illustrated in Fig.7.4Bf. The amplitude of this trh was 38mV whereas the coincident inward

Figure 7.5

Fertilization record of the responses observed in an egg fertilized in CalOK5. The patch pipette was filled with this solution diluted to 90%. A is a pen trace recording, the upper trace is the extracellular current and the lower trace is the intracellular potential record. Letters a-e refer to the oscilloscope pictures in B. The action potential is shown as an oscilloscope trace in Ba (lower trace) and at a faster sweep speed in Bb. The accompanying extracellular current recorded is shown in Ba (upper trace) and at a faster sweep speed in Bc. Arrows above the traces indicate the approximate time of the first sperm fusion. The egg was confirmed by histology to be dispermic, but the time of the second fusion is not known.


A
Sp 1
Current (pA)
in
out
4
0
4
8
Current clamp potential
(mV)
0
-40
-80
a-c
d
e
1.0
2.0
3.0
4.0
5.0
Time (mins)
B
a
Sp 1
b
Sp 1
c
Sp 1
d
e
Current (pA)
0
2
4
6
80mV
0.4sec
40mV
0.1sec
2pA
0.1sec
40mV
2pA
2sec

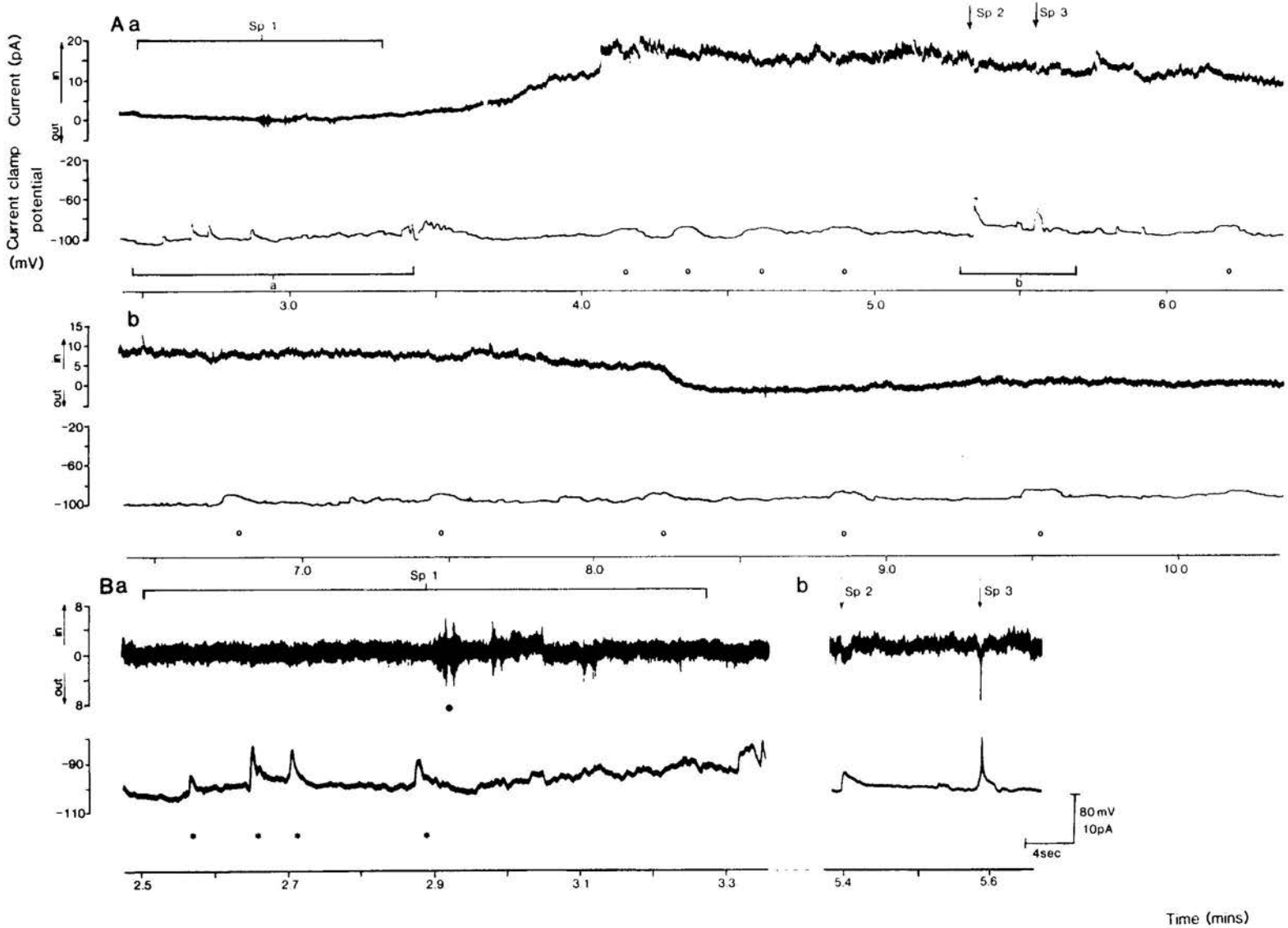
current on the extracellular current trace was only 0.8pA.

It is interesting that the trhs recorded prior to the trh spikes had after-depolarizations, whereas none of the trhs recorded after the trh spike, had any such responses.

In another egg fertilized after current clamping the egg at -78mV the first sperm fusion elicited an action potential (Fig.7.5A, 7.5Ba-c). This action potential had a predepolarization, observed on the intracellular record shown in Fig.7.5Ba (bottom) and Fig.7.5Bb. This predepolarization was coincident with an outward current on the extracellular current trace (shown in Fig7.5.Ba top and Fig.7.5Bc.) This action potential was followed by 23 trhs (the first five are shown in Fig.7.5A) superimposed on its plateau. On this occasion it was possible to see the inward current across the patch membrane, coincident with each trh. The first and the fourth trhs are also shown as oscilloscope pictures in Fig.7.5Bd and Fig.7.5Be. These trhs were superimposed on a gradual hyperpolarization of the current clamp potential. Before the first trh the current clamp potential was -38mV and that prior to the 16th trh (not shown) it was -51mV. The gradual hyperpolarization was observed as a slow inward current on the extracellular current trace (indicated by a gradual upward movement of the extracellular trace in Figure 7.5A). Although such a situation might also arise if the resistance between

Figure 7.6

Pen trace recording (A) and oscilloscope pictures (B) of responses recorded in an egg bathed in Cal0K5 and fertilized by three sperm. The timing of each fusion is indicated by arrows above each trace - Ab is a continuation of the record in Aa. Open circles indicate trds (A), the filled circle indicates an increase in noise (Ba) and the asterisk refers to the small depolarizations accompanying the first fusion (Ba). This egg was current clamped at -105mV prior to insemination. The patch pipette was filled with Cal0K5 diluted to 90%. Letters "a" and "b" on A refer to the corresponding oscilloscope pictures in B.


Aa
Sp 1
Sp 2
Sp 3
Current (pA)
in
out
20
10
0
-20
-60
-100
Current clamp potential (mV)
a
b
3.0
4.0
5.0
6.0
b
15
10
5
0
7.0
8.0
9.0
10.0
Ba
Sp 1
8
0
-90
-110
2.5
2.7
2.9
3.1
3.3
b
Sp 2
Sp 3
80mV
10pA
4sec
5.4
5.6
Time (mins)

the pipette tip and the bath gradually decreased.

The interpretation of the result of another experiment illustrated in Fig.7.6, is as follows. The resistance between the extracellular pipette tip and the bath decreased initially, possibly due to sperm motility and subsequently returned to it's initial level. Such changes in the resistance were also observed during intracellular recording experiments and have been interpreted as being due to an instability in the recording. During this experiment a poor estimate of the timing of the first fusion was obtained (Sp1). It occurred at some time between 2.5 and 3.3 minutes after sperm addition. This length of recording is shown as an oscilloscope trace (Fig.7.6Ba). During this period four depolarizing fluctuations were observed (marked by asterisks in Fig.7.6Ba) on the intracellular record. The first three of these were unaccompanied by any distinguishable response on the extracellular current trace. The last of these small depolarizations was almost coincident with an increase in noise at 2.9 minutes (marked by a filled circle below the extracellular current trace in Fig.7.6Ba). The second and third fusions (Sp2 and Sp3) were associated with an fsd and an action potential respectively (7.6Aa and 7.6Bb). The fsd and the sperm evoked action potential were both accompanied by an outward current across the patch, the latter also being associated with an outward capacitative current.

Table 7.1

Durations and amplitudes of the slow responses recorded in eggs fertilized at low membrane potentials. The amplitude of the responses recorded with intracellular electrodes (i.e. trhs and trh spikes)are expressed in millivolts and the amplitudes of the responses measured simultaneously with the extracellular pipettes, i.e. trh(I)s and trh spike(I)s are expressed in picoamps. The results are expressed in the form mean $\pm$ SD, (n=).

Table 7.2

Durations and amplitudes of the slow responses recorded in eggs fertilized at high current clamp potentials. Results are expressed in the form mean $\pm$ SD, (n=), but in cases where only a few results were available the actual values are quoted (shown in italics).

Type of recording electrode	DURATION (sec)		AMPLITUDES		
	trh or trh(I)	trh spike or trh spike(I)	trh or trh(I)	trh spike or trh spike(I)	amplitude of spike of trh spike or trh spike(I)
Intracellular	8±1 (13)	11±2 (5)	25±7 (13)mV	40±4 (5)mV	40±8 (5)mV
Extracellular	7±1 (10)	8±2 (5)	0.7±0.2 (5)pA	0.3±0.1(5)pA	3.7±1.6 (5)pA

TABLE 7.1

Type of recording electrode	DURATION (sec)				AMPLITUDES				
	trh or trh(I)	trh spike or trh spike(I)	trd or trd(I)	after depol^{n} or after depol^{n} (I)	trh or trh(I)	trh spike or trh spike(I)	Amplitude of spike of trh spike or trh spike(I)	trd or trd(I)	after depol^{n} or after depol^{n}(I)
Intracellular	6±3 (37)	10±1 (5)	9±2 (10)	3, 4	13±10(36) mV	19±7(5)mV	5, 26, 38 44mV	10±2(10) mV	4, 4mV
Extracellular	5±2 (11)	-	-	6, 6, 8, 10	1.1±0.6(11) pA	-	1·4, 2·0, 3·5, 3·5 pA	-	0·6, 0·6, 0·6, 0·7 pA

TABLE 7.2

After the first fusion trds were observed but none of these were seen on the extracellular current trace. All trds have been indicated by open circles below the intracellular pen trace in Fig.7.6A.

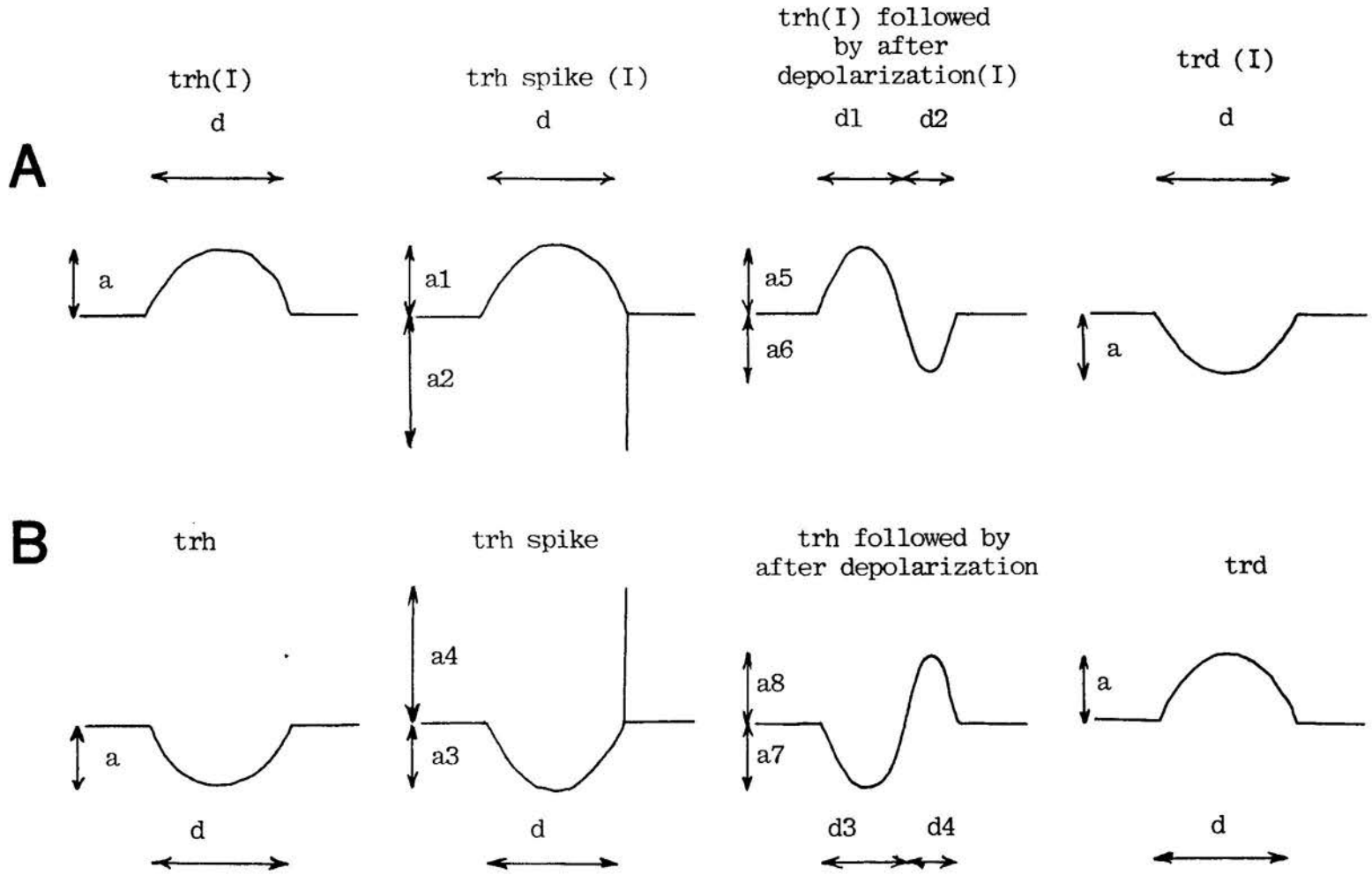
The extracellular current trace is interpreted as follows. Starting at about 3.5 minutes the resistance between the pipette and the bath gradually decreased, then decreased further rather suddenly at about 4.0 mins. Both these phases were associated with an increase in noise (possibly "rim" currents, i.e. ionic movement between the pipette interior and the bathing fluid.) The resistance then increased, returning almost to its initial value at about 8.3 mins (as the resistance increased the noise decreased). Unfortunately there is no evidence for this interpretation.

7.4 Analysis of the fertilization responses recorded with both intracellular electrodes and extracellular pipettes simultaneously (Tables 7.1 and 7.2)

Results of fertilization experiments in which the responses were monitored with both intracellular electrodes and extracellular pipettes have been divided into two groups. In one group are included the responses recorded in eggs fertilized at low membrane potentials and in the second group are those recorded in eggs at high current clamp potentials. These results have been summarized in Tables 7.1 and 7.2 respectively.

Figure 7.7

A schematic illustration of the various slow responses (see Chapter 5) recorded by an extracellular pipette (A) and an intracellular electrode (B). These responses are not drawn to scale. Letters "a" and "d" denote the amplitude and duration of each response respectively. The symbols a1, a2, a3 and a4 refer to the amplitude of a trh spike(I), spike of trh spike(I), trh spike and spike of trh spike respectively. Similarly a5, a6, a7 and a8 are symbols which refer to the amplitude of trh(I), after-depolarization(I), trh and after-depolarization respectively. The symbols d1 and d2, refer to the durations of the trh(I) phase and the after-depolarization (I) phase of a "trh(I) followed by an after-depolarization(I)". Similarly d3 and d4 are the durations of the trh phase and the after-depolarization phase of a "trh followed by an after-depolarization". The amplitudes of responses recorded with the extracellular pipette were measured in pA and those with the intracellular electrode in mV. Upward deflections on the extracellular record indicate inward currents. Although trd(I)s were not recorded, such responses have nevertheless been depicted in this diagram. Measurement of amplitudes and durations of the two phases of a "trh spike (I) followed by after-depolarization (I) responses" was made in a similar manner to that of "trh (I) followed by after-depolarization (I) responses".


A
trh(I)
d
a
trh spike (I)
d
a1
a2
trh(I) followed
by after
depolarization(I)
d1
d2
a5
a6
trd (I)
d
a
B
trh
a
d
trh spike
a4
a3
d
trh followed by
after depolarization
a8
a7
d3
d4
trd
a
d

The responses recorded by the intracellular electrodes have been called trhs, trh spikes and trds. The latter only being recorded in eggs current clamped at high potentials. Trhs were sometimes followed by after-depolarizations in eggs current clamped at high potentials. The responses recorded by the extracellular pipette coincident with these responses recorded by the intracellular electrode have been designated as trh (I), trh spike (I) and after-depolarizations (I). No trd (I) responses were recorded. In Figure 7.7, these responses recorded by the extracellular pipette (A) and the intracellular electrode (B) have been drawn schematically. These diagrams are not drawn to scale, their only purpose being to illustrate how the amplitudes and the duration of the various responses were measured (see legend to Fig.7.7).

The amplitudes of the currents measured by the extracellular pipette, are outward for the following responses:- amplitude of the spike of a trh spike (I) response (a2; this is believed to be an outward capacitative current), and after-depolarizations (I) (a6; these followed trhs and are interpreted as being outward capacitative currents). Trh (I)s and trh spike (I)s were observed as inward capacitative currents on the extracellular current trace.

The durations of trhs recorded by intracellular electrodes and the durations of trh(I)s recorded by

extracellular pipettes were comparable, for both "low membrane potential fertilizations" (Table 7.1) and "high current clamp potential fertilizations" (Table 7.2).

The durations of trh spike responses recorded intracellularly were significantly longer than trhs observed in low membrane potential fertilizations ($p<0.05$; Fisher Behrens test) and in high current clamp potential fertilizations ($p<0.01$; Fisher Behrens test). But there was no difference in the duration of trh spike(I)s and trh(I)s (recorded extracellularly) in eggs fertilized at low membrane potentials ($p>0.05$; Fisher Behrens test).

Trds were only observed in eggs current clamped at potentials more negative than about -80mV (see Chapters 5 and 6). Their durations were significantly larger than trhs ($p<0.01$; Fisher Behrens test), but no different to that of trh spikes ($p>0.05$; Fisher Behrens test).

No after-depolarizations were observed to follow trhs in eggs fertilized at low membrane potentials, but they were recorded in eggs fertilized at high current clamp potentials. The durations of these after depolarizations recorded by intracellular electrodes were 3 and 4 seconds each with an amplitude of 4mV whereas the duration of after depolarization(I)s recorded by extracellular pipettes were 6,6,8 and 10secs, which had amplitudes of 0.7, 0.6, 0.6 and 0.6pA respectively.

The mean amplitude of the extracellular current recorded during trh(I)s and trh spike (I)s observed after fertilization of low membrane potential eggs was 0.7±0.2pA (mean ± SD), n=5 and 0.3±0.1pA (mean ± SD) n=5 respectively.

Amplitudes (as recorded with intracellular electrodes) of trhs and trd spikes were less in eggs current clamped at high potentials than those observed in low membrane potentials. This would be expected since by definition an egg current clamped at a high potential is "closer" to the reversal potential for these slow responses. It is therefore somewhat surprising that the mean amplitude of the inward extracellular current accompanying trh(I)s is larger in eggs current clamped at high potentials compared to those observed in low membrane potential eggs. This is probably due to the small number (five) of trh(I)s recorded in low membrane potential fertilizations in this study.

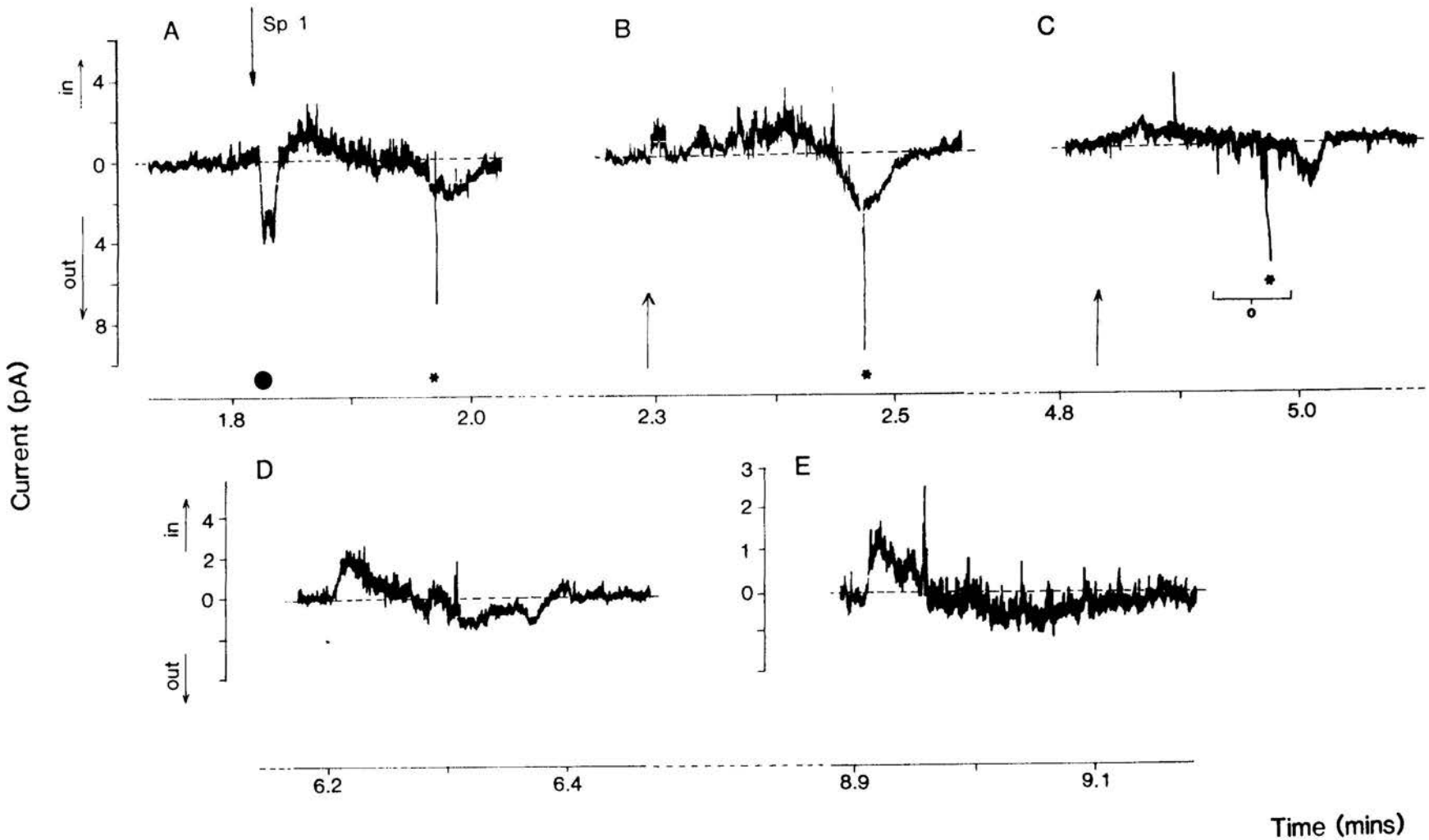
The reversal potentials of 13 trds recorded in the above experiments was -82±2mV (mean ± SD), and that of the 3 trhs was -74,-74 and -84mV.

7.5 Fertilization responses recorded by an extracellular pipette

In this section only the extracellular current was measured during the fertilization of zona-free hamster eggs, i.e. the eggs were not simultaneously impaled

Figure 7.8

Oscilloscope pictures of five of the responses recorded in an egg fertilized in Cal0K5 (patch pipette filled with TEA-10 CsCl-120). The arrow above the trace in A indicates the instant of sperm fusion which was associated with a predepolarization(I) (marked by filled circle below the trace). Asterisks in A and B refer to the outward capacitative currents and that in C refers to an outward current which may be either ionic or capacitative. Arrows below the traces in B and C indicate the time for the onset of the response. Open circle below the trace in C draws attention to the burst of outward channel activity. Dotted lines are drawn across each trace to indicate a "zero base line" from which the amplitudes of each phase of the response were measured.


A
Sp 1
B
C
in
4
0
out
4
8
1.8
2.0
2.3
2.5
4.8
5.0
D
E
in
4
2
0
out
3
2
1
0
6.2
6.4
8.9
9.1
Current (pA)
Time (mins)

with an intracellular electrode.

During such experiments eleven eggs were fertilized whilst bathed in Ca10K5 (in 8 of these the patch pipette filling solution was TEA-10 CsCl-120 and in the remainder it was Ca10K5 diluted to 90%), two in Ca10K25 (in both these the patch pipettes were filled with TEA-10 CsCl-120) and one in Sr10K5 (patch pipette filled with Ca10K5).

Five of the responses recorded in an egg fertilized in Ca10K5 (patch pipette filled with TEA-10 CsCl-120) are shown in Fig.7.8. The trh spike(I) response shown in A was believed to accompany the first sperm fusion. There was an outward current (marked by a filled circle in Fig.7.8A) with an amplitude of 4.2 pA and a duration of 1sec. Since an inward current across the patch (on the extracellular trace) was coincident with the hyperpolarization during a trh, then an outward current must be coincident with a depolarization. Therefore because the depolarization caused by the outward current preceded the trh spike (I) response it has been called a predepolarization. Returning to the convention adopted so far in this chapter - a predepolarization (a voltage change) is coincident with a predepolarization (I), recorded on the extracellular record (the latter being a current, called an outward current above). After this predepolarization (I) was an inward current (with an amplitude of 1.2pA and a duration of about 5secs) followed by an outward current, the latter having

superimposed on it a fast outward current (spike). The inward current has been interpreted as being due to the trh phase of the response and the slow outward current as the after-depolarization which followed it. The amplitude of this after-depolarization (I) was 1.8pA (i.e. larger than the inward current), and it's duration also about 5 seconds. The fast outward current (marked by an asterisk in Fig.7.8A) is the capacitative outward current due to the spike phase of a trh spike response. About 15 secs after the end of this response a second trh spike (I) was recorded, which is shown in Fig.7.8B. The onset of this response has been marked by the arrow below the trace in Fig.7.8B. The durations of the slow inward and outward currents were 9 and 4 secs respectively, whereas the amplitudes of the slow inward and outward currents were 1.4 and 2.6pA respectively. Again, the spike of the trh spike was observed as an outward capacitative current on the extracellular trace, marked by the asterisk in Fig.7.8B. This response emphasizes the approximate nature of the estimation of the duration of these responses, since the start of the trh (I) is sometimes taken as being the instant when the "noise" increases. Four further trh spike (I)s (not illustrated) were recorded at intervals of 16,17,28 and 30 secs, followed by the response shown in Fig.7.8C. A large transient outward current was observed in Fig.7.8C marked by an asterisk below the trace. It is

Table 7.3

Durations and amplitudes of successive responses recorded during fertilization of an egg bathed in Ca10K5. Patch pipette filled with TEA-10 CsCl-120. "Total duration" of a slow response is the sum of the duration of the after-depolarization (I) and either the trh(I) or trh spike(I) response.

Time of occurence of successive slow responses	trh (I)		after-depolarization (I)		trh spike (I)		"total duration" of slow response (seconds)
	Duration (seconds)	Amplitude (pA)	Duration (seconds)	Amplitude (pA)	Duration (seconds)	Amplitude (pA)	
1.2			5	1.5	9	0.3	14
2.6			6	1.5	6	0.4	12
3.0			3	1.8	8	1.0	11
3.3			4	2.0	5	0.8	9
3.6	7	1.4	6	2.6			13
4.0	5	2.0	6	1.4			11
4.6	4	2.4	7	1.4			11
5.6	3	2.0	3	1.3			6
6.7	5	2.0	7	1.4			12
7.0	5	2.0	7	1.2			12
8.0	4	2.5	6	1.0			10
9.0	5	3.0	5	0.5			10

TABLE 7.3

not possible to conclude whether this is an outward capacitative current, due to a calcium influx, or an outward ionic current due to channel opening in the patch. The amplitude of the slow inward and outward currents was 1.0 and 1.4pA. A burst of outward currents were also noted during this response (marked by an open circle in Fig.7.8C). All subsequent slow responses were trh(I)s, two of which are shown in Fig.7.8D and Fig.7.8E.

Ignoring small variations between successive slow responses, there appeared to be a gradual decrease in the amplitude of the after-depolarization (I) (e.g. compare Fig.7.8B and Fig.7.8E) as measured by the slow outward current, until no such response was discernible. The amplitude of slow inward currents of successive responses increased, indicative of an increase in the amplitude of trhs. In Table 7.3 is presented some actual data from an experiment similar to that discussed above. The results in Table 7.3 show that the amplitude of the slow inward current which accompanied either trh spike (I)s or trh (I)s increased from 0.3pA (response at 1.2mins) to 3.0pA (response at 9.0mins). Similarly the after-depolarization which accompanied each of these decreased from 1.5pA to 0.5pA. In this experiment also the first few slow responses were trh spike (I)s (4 in all) and the following 8 were trh(I)s. The total duration of each slow response, i.e. the sum of the duration of the after-depolarization (I) and either that of the trh(I),

or trh spike (I), was 11±12 secs (mean ± SD) n=12.

Of eight such experiments (in which eggs were fertilized in Ca10K5 and the patch pipettes were filled with TEA-10 CsCl-120) in five trh (I) responses followed trh spike (I)s, in two only trh spike (I)s were observed and in one only trh(I)s were observed. In no experiments were trh spike (I)s observed after trh (I)s.

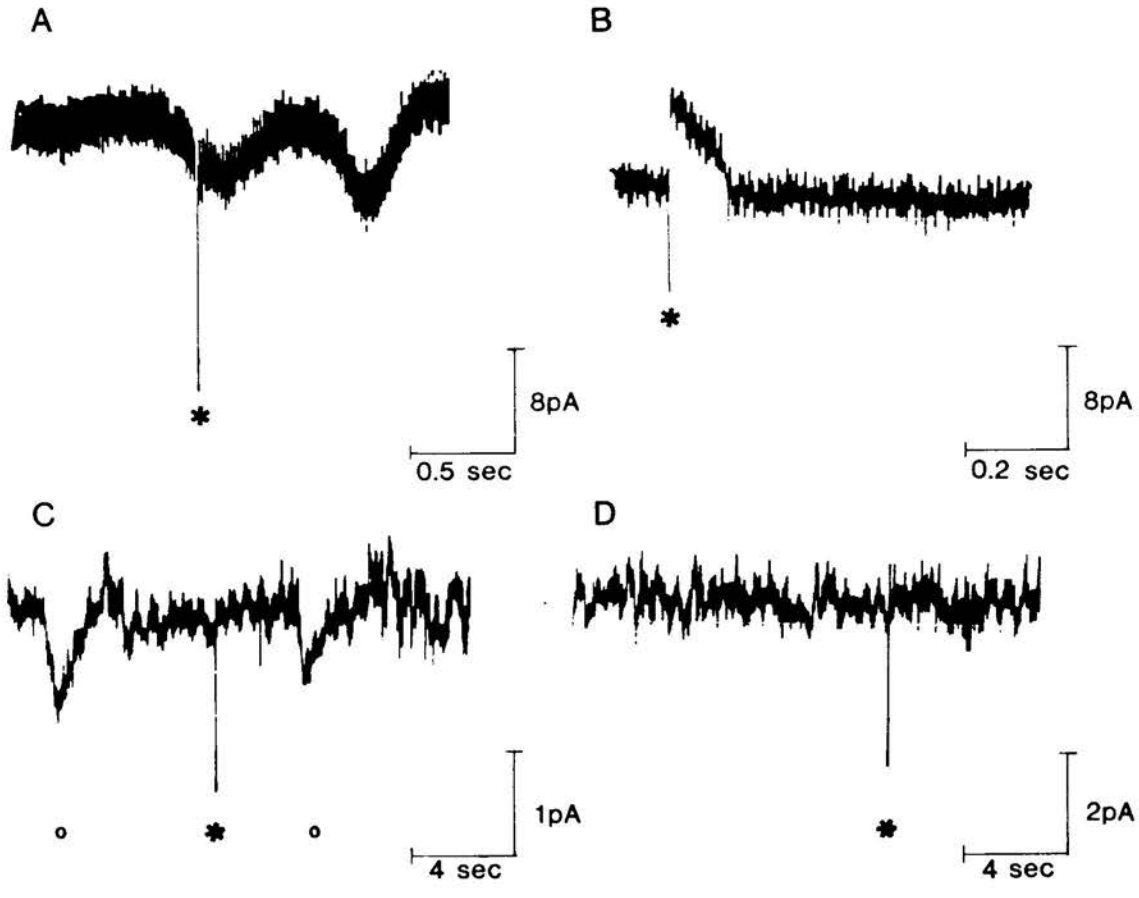
Predepolarization (I)s (i.e. slow outward currents occurring at the onset of the slow responses) were observed on 8 occasions. In one egg predepolarization (I)s accompanied the first three slow responses, in three other eggs a predepolarization (I) accompanied the first slow response (one of which is shown in Fig.7.8A), in one egg a predepolarization (I) was observed at the third and in another at the fourth slow response. Only in the example illustrated was a sperm fusion observed to accompany the predepolarization (I). In the remaining seven predepolarization (I)s, such visual confirmation was not possible for technical reasons.

In the one egg fertilized in Sr10K5, trhs were recorded. Three eggs were fertlized in Ca10K5, with patch pipettes containing Ca10K5 diluted to 90%. In two of these just trh spikes were recorded, and in another egg a trh response was recorded after a trh spike.

In the two eggs fertilized in Ca10K25 (in both cases the patch pipette was filled with TEA-10 CsCl-

Figure 7.9

Oscilloscope pictures of "unexplained responses" observed during fertilization of eggs bathed in Ca10K25 (A and B) and Ca10K5 (C and D). In all these the patch pipettes were filled with TEA-10 CsCl-120. Asterisks denote "possible" fast outward capacitative currents (A -D) and open circles refer to "possible" slow outward capacitative currents (C). The latter are believed to be analogous to depolarizing fluctuations of the membrane potential observed with intracellular electrodes.


A
8pA
0.5 sec
B
8pA
0.2 sec
C
1pA
4 sec
D
2pA
4 sec

120), no trh or trh spike responses were observed. In one of these the response shown in Fig7.9A was observed and in the other that shown in 7.9B. Unfortunately these were not confirmed visually to have occurred at the time of fusion, furthermore the eggs were confirmed histologically to be polyspermic.

But it could be argued that these are fast outward capacitative currents (associated with a spike of an action potential) followed by a slow outward current (Fig.7.9A) or an inward current (Fig.7.9B). The other argument is that in these two eggs the membrane potential was close to the reversal potential of slow responses, i.e. trhs and trh spikes. The reversal potential of slow responses in Cal0K25 is expected to be about -50mV (see Chapter 5), and therefore it may be concluded that the membrane potential of these two eggs was low.

Furthermore during these experiments, fast outward capacitative currents were frequently recorded,such as those shown in Fig.7.9C and Fig.7.9D (marked with asterisks below the trace). Unfortunately the possibility that they may be artefactual has not been ruled out. Also not all these outward capacitative currents were correlated visually with sperm fusion. In the experiments that such visual correlation was "apparently possible", they were so frequent that the results could not be considered reliable.

When the eggs were current clamped at high potentials (as in Chapters 5 and 6) then depolarizing

fluctuations were frequently recorded. Similarly when the eggs described in the present chapter were current clamped at high potentials, outward currents were recorded across the patch. These would theoretically be due to an inward ionic current across the egg membrane, causing an outward capacitative current across the patch membrane (into the pipette). Two such outward currents are observed in Fig.7.9C (marked with open circles). It may be argued that the predepolarization (I)s (discussed above) are in fact such outward currents. But it was observed that predepolarization (I)s occurred only in the early part of each experiment. As mentioned above they were seen to be associated with the first slow response, on four occasions (out of eight). This idea is supported by the fact that most sperm fusions occur in the early part of an experiment (soon after insemination of the recording chamber), as observed in Chapters 5 and 6. It can be argued that as the egg depolarizes (which was often observed during intracellular recording of fertilizations in eggs current clamped at high potentials), then depolarizing fluctuations decrease in frequency and amplitude, and this is why outward currents (as in Fig7.9C) are not observed in the latter part of experiments.

7.6 Analysis of fertilization responses recorded with extracellular pipettes only (Tables 7.4 & 7.5)

Table 7.4

Durations of the responses recorded with extracellular pipettes during fertilization of eggs bathed in Ca10K5 and Sr10K5. Results expressed either as mean ± SD (n=) the actual values are quoted (in italics).

Table 7.5

Amplitudes of the responses recorded with extracellular pipettes during fertilization of eggs bathed in Ca10K5 and Sr10K5. The results expressed either as mean ± SD (n=) or the actual values are quoted (in italics).

Bathing solution	DURATIONS (seconds)							
	Patch pipette filled with TEA-10 CsCl-120				Patch pipette filled with Cal0K5 (diluted to 90%)			
	trh(I)	trh spike(I)	after depol^{n} (I)	predepol^{n} (I)	trh(I)	trh spike(I)	after depol^{n} (I)	predepol^{n} (I)
Cal0K5	5±1 (31)	7±2 (38)	5±2 (52)	1, 1	5	4, 5, 7, 9	5, 6, 6	
Sr10K5					3, 4		4	

TABLE 7.4

Bathing Solution	AMPLITUDES (pA)									
	Patch pipette filled with TEA-10 CsCl-120					Patch pipette filled with Cal0K5 (diluted to 90%)				
	trh(I)	trh spike(I)	amplitude of spike of trh spike(I) response	after depol^{n} (I)	predepol^{n} (I)	trh(I)	trh spike(I)	amplitude of spike of trh spike(I) response	after depol^{n} (I)	predepol^{n} (I)
Cal0K5	2.0±1.6 (30)	1.0±0.7 (34)	5.4±3.3 (39)	1.2±0.7 (57)	2.2±1.2 (6)	0·8	0·8, 1·4, 2·0	1·0, 2·2, 4·0 5·2	0·4, 0·8, 2·4	
Sr10K5						1·6, 4·4			1·2	

TABLE 7.5

Most of the results of this group of experiments were obtained in eggs fertilized in Cal0K5 (the patch pipettes being filled with TEA-10 CsCl-120). These results are discussed below, but all the results are summarized in Tables 7.4 and 7.5.

Of the 69 slow responses [trh(I)s or trh spike (I)s] recorded, after-depolarization (I)s were observed in 57 of them, whereas in the group of experiments discussed in Section 7.3 (in which there was an additional intracellular electrode) in only four trh(I)s out of 42 trh(I)s and trh spike(I)s were after -depolarization (I)s observed. The current clamp potential (measured by the intracellular electrode) prior to the onset of each of these trh(I)s with after -depolarization (I)s was -40mV, -44mV, -50mV and -50mV. Similarly in two trhs out of 42 trhs (recorded with intracellular electrodes during the "dual recording experiments") and trh spikes recorded were after-depolarizations noted. The current clamp potential prior to the onset of each trh followed by an after -depolarization was -68mV and -69mV.

There was no significant difference in the durations of trh(I)s and after-depolarization (I)s, but trh spike(I)s were significantly longer than trh(I)s and after-depolarization(I)s (in both cases $p < 0.01$; Fisher Behrens test).

There was no significant difference in the amplitude of trh spike(I)s and after-depolarization(I)s ($p>0.05$; Fisher Behrens test). But trh(I)s were larger

than trh spike(I)s ($p<0.01$; two sample t test) and after-depolarization(I)s ($p<0.05$; Fisher Behrens test). The mean amplitude of trh(I)s was comparable to that of predepolarization(I)s. It was of interest to compare the results of the above group of experiments with those obtained in eggs current clamped at high potentials prior to fertilization (summarized in Table 7.2). The durations of trh(I)s observed in experiments with just extracellular pipettes was the same as that in experiments with an additional intracellular electrode (5 ± 1 secs, n=31 and 5 ± 2 secs, n=11 respectively). But the amplitude of trh(I)s was significantly larger in experiments with just the extracellular pipette compared to the "dual recording" experiments ($p<0.05$; Fisher Behrens test: 2.0 ± 1.6pA, n=30 and 1.1 ± 0.6pA, n=11 respectively - see Table 7.2).

The amplitudes of trh(I)s and trh spike(I)s recorded with just extracellular pipettes were larger than those recorded in eggs with both intracellular and extracellular electrodes (eggs fertilized at low membrane potentials - Table 7.1).

The amplitude of the spike of trh spike(I)s was not significantly different when recorded in experiments with just extracellular pipettes to that recorded in the dual recording experiments at low membrane potentials, ($p>0.05$; Fisher Behrens test) although the mean amplitude was certainly greater in the former - 5.4pA compared to 3.7pA.

Two criticisms of the above mentioned comparisons are that firstly durations of responses measured from extracellular records may be underestimated, because amplitudes of the slow responses is so small, often just being observed as an increase in noise. Secondly, the amplitudes of the responses are small and hence the possible error in measurement is large. Furthermore the solution used to fill the patch pipettes in the majority of experiments described in Section 7.5 was TEA-10 CsCl-120, whereas that in the dual recording experiments (Section 7.3) was Cal0K5 diluted to 90 per cent.

7.7 Discussion

Differences were observed in the fertilization responses recorded with extracellular pipettes in (i) dual recording experiments (i.e. both intracellular electrode and extracellular pipette) and (ii) experiments with an extracellular pipette only. These include:-

a) A much larger proportion of slow responses (57 out of 69) showed after depolarization (I)s in the "extracellular only" experiments. In the dual recording experiments 4 out of 42 slow responses showed after-depolarization (I)s (see 7.6). Since the current clamp potential prior to the after-depolarization (I)s in the dual recording experiments was in the range -40 to -50mV, this range may be a crude approximation to the membrane potential of the eggs in the

"extracellular only" experiments.

b) Amplitude of trh (I)s was significantly larger in the "extracellular only" experiments compared to the dual recording experiments at high current clamp potentials (see 7.6). A possible explanation for this is that the transpatch potentials of the eggs in the "extracellular only" experiments was more positive than the current clamp potentials in the dual recording experiments (about -80mV). Another possibility is that microelectrode insertion into the egg (in the dual recording experiments) has created a low resistance shunt which attenuates the size of the response observed in these eggs.

c) The amplitudes of trh (I)s and trh spike (I)s were larger in the "extracellular only" experiments than in the dual recording experiments at low membrane potentials. Explanations for this are, either the response is smaller in the dual recording experiments because of damage caused by impalement (see b above), or the transpatch potentials in the "extracellular only" experiments were more positive than the membrane potentials of the eggs in the dual recording experiments (about -30mV).

The term transpatch potential has been used above, and not the term membrane potential because the membrane potential of the egg is not equal to the potential difference across the patch (7.2 and Fig.7.2). An outward capacitative transient current

was observed on the extracellular record, in synchrony with anode break and action potential spikes which may be explained by:-

a) The transpatch potential is more negative than the membrane potential of the egg, thus causing a current to flow from the egg membrane (which is positive relative to the patch because of the calcium influx during the spike) into the patch.

b) If the membrane within the pipette is less excitable than that of the remainder of the egg (i.e. threshold for calcium channels is more positive), then current will flow inward through excitable channels in the spherical portion of the egg and outward through the relatively less excitable membrane within the pipette.

c) The calcium channels in the patch are inactivated, as might occur if the patch was depolarized.

The slow responses during fertilization of zona-free hamster eggs have been shown to be due to a calcium activated potassium conductance (Igusa & Miyazaki, 1983; Georgiou et al., 1983; see Chapter 5). It is assumed that cytosolic calcium rises uniformly over the inner surface of the egg membrane. (Although this may not strictly be true because a portion of the egg membrane is sucked into the pipette). Then if the egg and the patch are isopotential, the whole of the egg membrane (including patch) should undergo potassium channel opening, and an outward ionic potassium current should be observed across the patch. But instead a slow inward current was observed, which is indicative

of an anisopotentiality between the egg membrane and the patch. This slow inward current must therefore be capacitative. This anisopotentiality may be caused by:-

a) the potassium channels in the patch are not activated by the rise in calcium initiated by the sperm. Either their threshold for opening is raised or the cytosolic calcium does not reach threshold levels for such a response, in the vicinity of the patch membrane.

b) the potassium channels in the patch are inactivated, possibly due to a physical distortion of the channels caused by the formation of a cell attached patch. But this is not in agreement with the results showing potassium channels in Chapter 3.

c) the patch is depolarized relative to the rest of the egg membrane. Hence when potassium channels open in the rest of the egg membrane causing a hyperpolarization (the patch is relatively depolarized), a slow capacitative inward current is recorded.

The possible reasons for observing a fast outward capacitative current in synchrony with the spike of a trh spike are the same as those mentioned above with regard to spikes of anode break responses and action potentials.

The fact that trh spikes were observed frequently in the experiments described in this chapter, may be an indication that the resistance of the seal around the

microelectrode and the egg membrane was larger than in the intracellular recording experiments described previously, i.e. the measured input resistance of the egg is higher. This would mean that for a given current flow across the egg membrane the rate of change of potential would be greater. Hence, the rate of repolarization of a trh (actually the rate of depolarization) may be greater in these experiments with extracellular pipettes compared to those with just intracellular electrodes. This greater rate of repolarization may be sufficient to overcome the calcium channel inactivation and hence cause a spike. No measurements were made in the present study to compare any possible differences in such rates of repolarization of trh spikes and trhs, but this merits further investigation.

The greater seal resistance (which in general would mean a greater recorded membrane potential) may be indicative of a greater stability of recording introduced by the presence of the patch pipettes, in the dual recording experiments, and furthermore, the absence of a leak pathway in the extracellular only experiments. It is interesting that although no trh spikes were observed in the experiments described in Chapter 5 and Chapter 6, a response which is similar was noted in an experiment illustrated in Fig.5.9 (see Fig.5.9Ad, Ae, Ag and Ah). Interestingly this result was of a fertilization of a high membrane potential egg (see 5.4) Furthermore, trhs recorded in this egg had

after-depolarizations (Fig.5.9Aj).

In the "extracellular only" experiments since the amplitude of the slow inward current increased in successive responses, then the amplitude (in terms of potential) of the slow responses must be increasing. Therefore fertilization must be accompanied by a depolarization, whereas previous fertilizations of low membrane potential eggs with intracellular electrodes has indicated a hyperpolarizing shift (Igusa & Miyazaki, 1983; Figures 5.1 and 5.2). In the fertilization experiments described of eggs, at high current clamp potentials, a depolarizing shift was generally (although sometimes after fsds and sperm evoked action potentials a gradual hyperpolarization was noted) observed (5.8). Therefore it is likely that the membrane potential of eggs (without intracellular electrodes) is higher (more negative) than those impaled with microelectrodes, i.e. there is a leak pathway introduced by microelectrode insertion.

Therefore to summarize, these experiments with extracellular pipettes have revealed the presence of yet another type of response (trh spike). Furthermore after-depolarizations are more frequent and there is a depolarizing shift of the potential after fertilization. These indicate that the membrane potential of the egg during "extracellular only" experiments is closer to the true resting potential of the egg (i.e. in the absence of a leak).

The extracellular pipettes used in the present study had resistances of 1-3 MΩ and internal diameters of about 2μm. In order to increase the amplitudes of the slow currents observed, it would be advisable in future experiments to use larger pipettes, and therefore sample larger areas of the egg membrane. In a similar study by Whitaker and Steinhardt (1983) on sea urchin eggs, the authors used pipettes with resistances of 0.1 to 0.3 MΩ and tip diameters up to 35 μm (the diameter of sea urchin eggs is nearly twice that of the preparation used in the present study).

Assuming that the area of the patch is $3x10^{-8}$ cm^2 (approximate area of circle of diameter 2μm; this being the internal diameter of the tip of the patch pipette) and assuming that the egg membrane has unit capacitance of $1\mu F$ cm^{-2}, then capacitance of the patch equals $3x10^{-2}$pF. The maximum rate of change of potential during the upstroke of the electrically evoked action potential was found to be $3Vs^{-1}$ (Georgiou et al., 1984). Therefore the maximum capacitative current across the patch, during an electrically evoked action potential is 0.09pA. This should be approximately equal to the amplitude of the spike of a trh spike (I) response, referred to in Tables 7.1, 7.2 and 7.5. The "amplitude of the spike of a trh spike (I) response" was found to be 5.4 ±3.3pA (n=39; Table 7.5) in the "extracellular only" experiments. The much smaller calculated current may be due to:-

a) an underestimation of the patch area, since some of

the egg membrane is sucked into the pipette.

b) an underestimation of the maximum rate of change of potential of the electrically evoked action potential. This could for example be caused by a leakage around the microelectrode.

c) underestimation of the specific membrane capacitance.

If the maximum amplitude of a trh response is assumed to be 40mV, then in an egg with an input resistance of 200MΩ, the current flowing across the whole of the egg membrane to produce this response must be 0.2×10^{-9}A. Then assuming a surface area of the egg of 2×10^{-4} cm^2 (a value of 6.6×10^{-4} cm^2 is not assumed as in the discussion in Chapter 5, because any underestimation of the total area of the egg due to microvilli would also apply to the calculated area of the patch - hence the "underestimation factor" would cancel out during the calculation), the current flowing across the patch is calculated to be 0.03pA. This current may be underestimated, due to an underestimation of the patch area caused by suction of the egg membrane into the pipette. It is therefore not surprising that on many occasions it was not possible to detect trh (I)s in synchrony with trhs.

CHAPTER 8 GENERAL DISCUSSION

In Chapters 3 and 4, ignorance of the true resting potential made some of the interpretations of results more difficult. In the present study there have been indications which suggest that the true resting potential of zona-free hamster eggs may in fact be higher than the values usually recorded with intracellular microelectrodes. These are listed below:-

a) inward channels were recorded on cell attached patches at zero pipette potentials which could have been either potassium or chloride (3.7 and Fig.3.10B). Furthermore calcium channels were recorded at zero pipette potentials. These are inactivated at potentials more positive than -60mV (approximately).

b) five eggs were found to be electrically excitable at rest, i.e. their membrane potentials were more negative than the threshold of the electrically evoked action potential (4.1, Table 4.1 and Fig.4.1D).

c) it was possible to evoke an action potential during whole cell recording. But these experiments need to be repeated with well defined Ca-EGTA buffers in the pipette filling solutions.

d) one of the whole cell recordings indicated a membrane potential of 52mV and an input resistance of 1030 MΩ(after correction). The seal resistance prior to breaking the patch in this example was 30GΩ. But such a seal resistance was obtained only once.

e) fertilization of a high potential egg (Fig.5.9) was recorded electrically as a sperm evoked action

potential followed by (i) responses which are reminiscent of trh spikes and (ii) trhs followed by after depolarizations.

f) recordings with extracellular patch pipettes revealed trh spikes (Chapter 7).

g) prominent after depolarizations were observed on most of the slow responses recorded during fertilization in the "extracellular only" experiments (Chapter 7).

h) after fertilization the amplitude of the slow responses increased, and the amplitude of the after depolarization decreased progressively indicating a gradual depolarization (7.6, Fig.7.8, Table 7.3).

It is possible that the enzymic treatment used in the preparation of the zona-free hamster eggs may be damaging the egg membrane. Hirao and Yanagimachi (1978) found that treatment of hamster eggs with trypsin or a variety of other enzymes did not impair fertilization of these eggs. But it should be remembered that the block to polyspermy in hamster eggs is at the level of the zona, and for this reason any damage to the membrane (with regards to the prevention of sperm fusion) may not be noted. By contrast the ability of mouse eggs to fuse with sperm is reduced when the zona is removed by proteases (Wolf, Inoue & Stark, 1976). But in mouse eggs the block to polyspermy resides at the egg membrane, hence any damage to it by enzymic treatment is manifested as a

reduced sperm penetration rate. Furthermore, McCulloh et al. (1983) on their study on rabbit eggs used a combination of mechanical and enzymic treatments to isolate zona-free eggs. It may therefore be better to remove the zona of hamster eggs by mechanical or a combination of mechanical and enzyme treatments, in future experiments. Although this may impose further limitations on the seal resistance obtained between patch pipettes and the egg membrane.

In the study by McCulloh et al. (1983) the rabbit eggs were naturally ovulated, and not obtained by superovulation induced by hormonal injections. Moor, Osborn & Crosby (1985) have reported that 28 % of PMS (this is the same hormone as used in the present study to stimulate follicular growth) treated sheep (oocytes showed changes in the pattern of protein synthesis which are normally associated with maturation. This may be a reason to prompt a few comparative experiments between naturally ovulated and superovulated hamster eggs.

The phenomenon of "rundown" observed in chromaffin cells has already been discussed in 3.8 (Fenwick et al., 1982b). But these authors observed that rundown could be accelerated by giving voltage pulses (50ms at 2Hz) to voltages where large calcium currents were induced. Since, during most of the intracellular recording experiments described in the present study, anode break spikes were elicited "en route" to obtaining a stable membrane potential, it may be argued that such a calcium influx may also cause a similar

rundown in the eggs. Furthermore if a substantial leak existed around the electrode tip at the instant of impalement, or at any time thereafter, then a calcium influx through this pathway may also have attributed to a rundown. During some impalements this calcium influx is seen electrically as an activation of the potassium conductance (Fig.4.1C). In the remainder, the changes in resistance observed after impalement (which have been interpreted as being alterations in the seal resistance around the microelectrode) may in fact be the sum of the resistance changes caused by (i) activation of the calcium activated potassium conductance and (ii) the alteration in the seal resistance between the electrode tip and the egg membrane. These two processes are antagonistic in terms of resistance changes. Therefore if the rundown caused by calcium influx is faster, than the time required for the egg membrane to seal around the electrode tip "maximally", then the true membrane potential will not be observed. It is interesting that in the impalement record of a high membrane potential egg illustrated in Fig.4.1D, the high membrane potential level was attained very quickly - in less than 3 minutes. Furthermore the number of anode break responses evoked was very small (about 10) in comparison to other impalements.

The reason for such a prominent possible rundown of mammalian eggs could be related to the fact that

(a) they have calcium activated potassium channels and (b) they have little or no sodium permeability (Okamoto et al., 1977). Whereas in echinoderms and echiurans there is a large sodium permeability. Indeed the amplitude of the positive going phase of the fertilization potential in both echinoderms and echiurans is due to a major permeability increase to sodium. No rundown has been observed with regard to sodium channels (Fenwick et al., 1982b). The fertilization potential in amphibians is due to a chloride permeability increase. There have been no reports on calcium activated potassium conductances in echinoderms echiurans or amphibians.

The outward potassium channels discussed in 3.3 and 3.4 are likely to be calcium activated potassium channels, the opening of which is responsible for the slow responses observed after fertilization. Calcium activated potassium conductance systems have been implicated in the regulation of repetitive activity in a wide variety of vertebrate and invertebrate neurones (Meech, 1978). Calcium influx during an action potential would tend to activate these channels hence causing an outward potassium current. Depolarization would make this hyperpolarizing current larger due to the greater driving force. If the calcium buffering capacity of the cell is influenced by the metabolic state, then the calcium activated potassium conductance would form a link between metabolism and membrane excitability (Meech, 1978).

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APPENDIX A INTRACELLULAR RECORDINGS DURING HOMOLOGOUS FERTILIZATIONS OF ZONA-FREE HAMSTER EGGS

A.1 Intracellular recordings during homologous fertilizations of zona-free hamster eggs, fertilized at low membrane potentials

A.2 Intracellular recordings during homologous fertilizations of zona-free hamster eggs, bathed in normal solution, current clamped at high potentials

A.3 Intracellular recordings during homologous fertilizations of zona-free hamster eggs bathed in high potassium solution, current clamped at high potentials

A.4 Discussion.

The following results are those of fertilizations performed in the following bathing solutions: Ca2K5 and Ca2K25. All of these were homologous fertilizations of zona-free hamster eggs. The experiments were identical to those described in Chapter 5 of this study (i.e. electrical events during fertilization were monitored using intracellular electrodes) . They were not included in the data shown in Chapter 5 because the current clamp potential and the input resistance registered by the amplifier was later found to be in error (due to amplifier malfunction). All these experiments were performed using the WPI amplifier, Model KS700, Dual Microprobe System. This amplifier when tested was found to have a substantial leakage current (as high as 100pA). Therefore the amplifier had essentially behaved as a current clamp. What was at the time thought to be the "membrane potential" of the egg was in fact the "current clamp potential". But because the leakage current varied with the resistance across its input (i.e. the input resistance of the egg), it was not possible to apply a "correction factor" to the measured current clamp potential in order to estimate the membrane potential of an egg. In addition the amplifier was found to have been over-estimating the input resistance of the egg. Hence for this particular group of experiments no attention has been paid to the measured input resistance (the range for which was 50 to 1000MΩ).

It is emphasized that all experiments described in the remainder of this study were performed using another amplifer (Dagan Model 8100 Single Electrode System) and in experiments requiring two electrode impalements, this was used in addition to another amplifier - WPI Microprobe System, Model M707.

This particular group of experiments was therefore quantitatively in error (in terms of input resistance and current clamp potential values), but was felt to be qualitatively correct and has therefore been included in this Appendix. In experiments where the measured input resistance (with the faulty amplifier) was less than about 150MΩ, the measured potential and input resistance was equal to the true value, i.e. the measured potential was the membrane potential of the egg and not the current clamp potential. Since low input resistances were associated with low membrane potentials, then the group of experiments performed on low membrane potential eggs were not in error. These are described in A.1.

In Chapters 5 and 6 an indication of the relative ease of fertilization of an egg, in various bathing solutions, was given by the ratio of the number of successful fertilizations to the number of intracellular recordings. No such fraction has been included in this appendix because these experiments were made at the beginning of this study when our experience of capacitation of sperm was meagre.

Another reason was that at that time it was not fully appreciated, how the age of the egg (time post - HCG injection), could govern whether or not the egg was fertilized. The eggs were at that time being used up to 20 hours after the HCG injection.

A.1 Intracellular recordings during homologous fertilizations of zona-free hamster eggs, fertilized at low membrane potentials

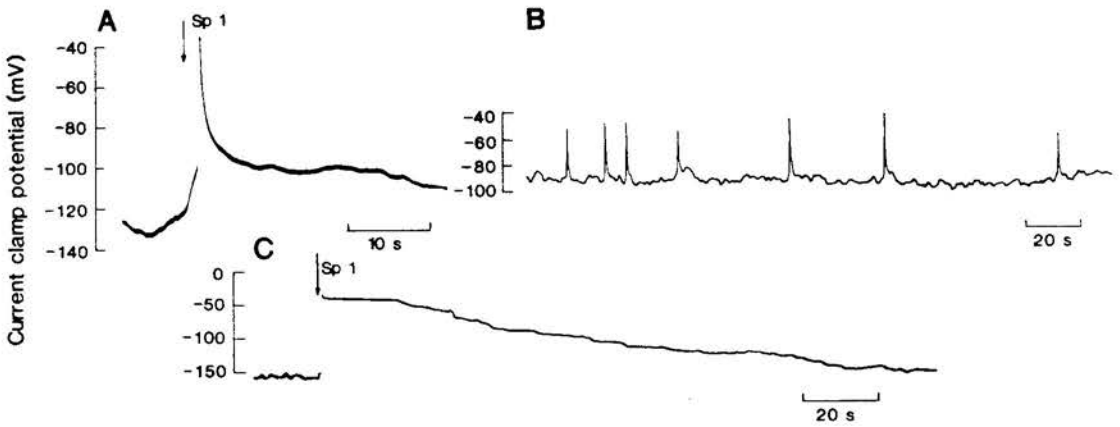
In eggs with low membrane potentials, fertilization was accompanied by trhs, as was observed by previous workers (Miyazaki & Igusa, 1982; Igusa et al, 1983). Trhs were observed in eighteen eggs bathed in normal solution (Ca2K5) and three eggs bathed in Ca2K25. Consistent with the idea that trhs are caused by a calcium activated potassium conductance, was the finding that the trhs observed in Ca2K25 were smaller than those observed in normal solution (Ca2K5).

A.2 Intracellular recordings during homologous fertilizations of zona-free hamster eggs, bathed in normal solution, current clamped at high potentials

Fig.A.1A and B show respectively a sperm evoked action potential following fusion and some relatively brief action potentials recorded later in the same egg. Zero time was taken as the instant of sperm addition to the bathing solution. Ten action potentials were observed at the following times: 0.7, 6.7, 6.8, 15.9, 16.2, 16.3, 16.6, 17.3, 17.9 and 19.0 minutes. With

Figure A.1

A is an oscilloscope picture of the first sperm evoked action potential recorded in an egg bathed in normal solution. This action potential accompanied a sperm fusion occurring 7 minutes after addition of the sperm to the chamber containing the impaled egg. B is a pen trace recording of a group of brief action potentials recorded shortly after the sperm evoked action potential shown in A. The first of this group of brief action potentials occurred 15.2 minutes after the one shown in A. C is a pen trace recording of the first sperm evoked action potential in another egg which accompanied the first sperm fusion. A further seven brief action potentials (similar to those shown in B) occurred during a period of 44 minutes after the first shown in C. Histological observations confirmed that the eggs were fertilized. Arrows indicate the times of the sperm fusions.


A
Sp 1
-40
-60
-80
-100
-120
-140
10 s
B
-40
-60
-80
-100
20 s
C
0
-50
-100
-150
Sp 1
20 s
Current clamp potential (mV)

the limited resolution of the methods used, it was not possible to determine the interval between fusion and the onset of depolarization causing the first sperm evoked action potential, but it is probably less than one second. The other action potentials were brief by comparison with the first; the last seven of the series of brief action potentials are shown in Fig.A.1B. Such a series of brief action potentials was observed to follow the first sperm evoked action potential (which accompanied sperm fusion, whereas the brief action potentials were not associated with sperm fusion) in one other egg fertilized in Ca2K5. Measurements from oscilloscope pictures of such brief action potentials indicated that they consisted of "spikes" of duration 500ms superimposed on depolarizations lasting 2.4±1.2 sec; mean ± SD, n=14.

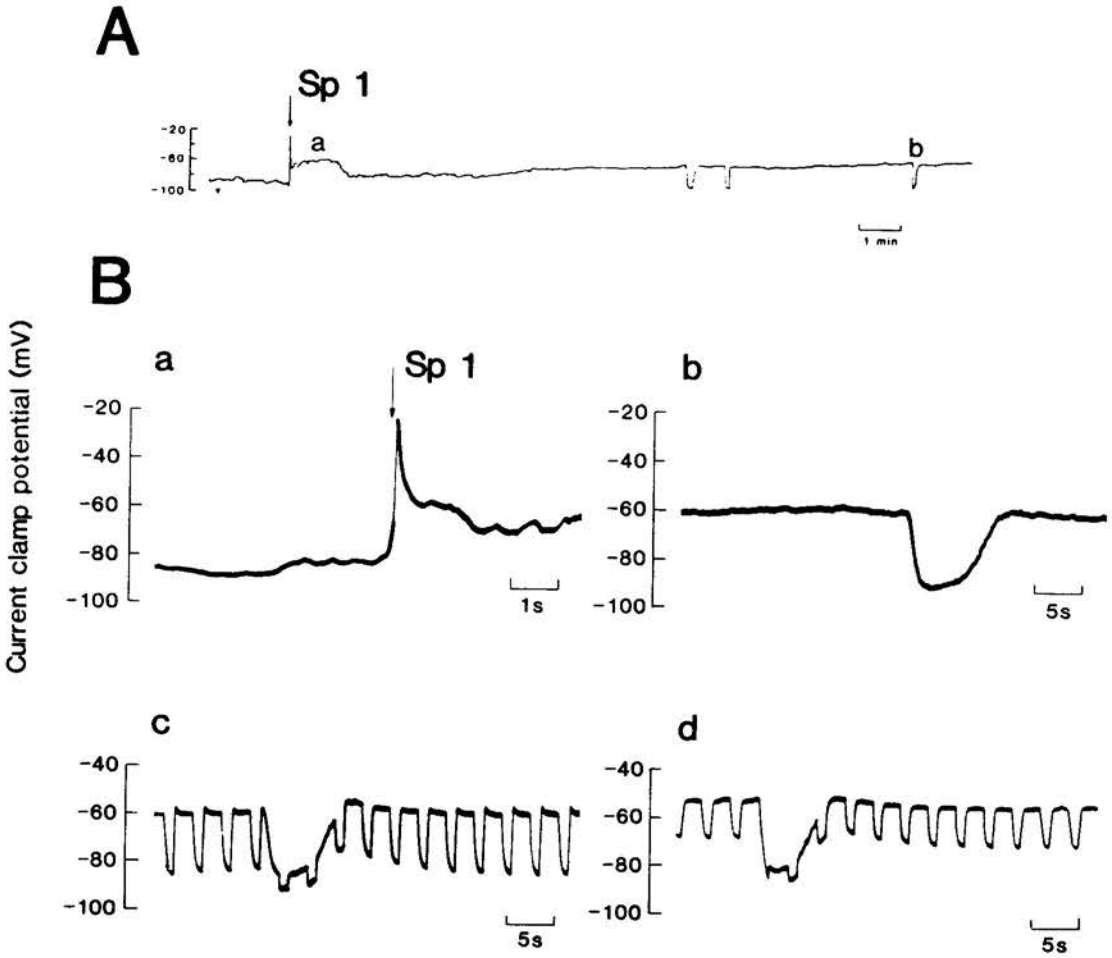
An intracellular recording from another egg shows the first sperm evoked action potential which accompanied the first sperm fusion (Fig.A.1C).

The range of durations of plateaux of sperm evoked action potentials observed in this study was 20 seconds to about 200 seconds (-66±78 seconds; mean ± SD, n=15). Seven sperm evoked action potentials had so called permanent plateaux, i.e. the potential did not return to the initial current clamp potential.

Occasionally spontaneous action potentials were observed in eggs current clamped at high potentials (see also Georgiou et al, 1984). In twelve such eggs

Figure A.2

A is a pen trace recording of a sperm evoked action potential followed by trh responses (the first three of which are shown) recorded in an egg fertilized in Ca2K5. The sperm evoked action potential is shown as an oscilloscope picture in Ba. The third trh shown in A is also shown in Bb. The trhs were superimposed on a gradual depolarization, observed after the action potential. Oscilloscope pictures Bc and Bd are further examples of trhs observed in another egg in which the sperm evoked action potential was again followed by trhs superimposed on a depolarizing shift. The downward deflections in Bc and Bd are due to small hyperpolarizing current pulses passed through the recording microelectrode (0.1nA, 1sec, 0.35Hz). The reversal potentials of the responses shown in Bc and Bd are -101mV and -97mV. Letters "a" and "b" in A refer to the corresponding oscilliscope picture in B.


A
Sp 1
-20
-60
-100
a
b
1 min
B
Current clamp potential (mV)
a
Sp 1
-20
-40
-60
-80
-100
1s
b
-20
-40
-60
-80
-100
5s
c
-40
-60
-80
-100
5s
d
-40
-60
-80
-100
5s

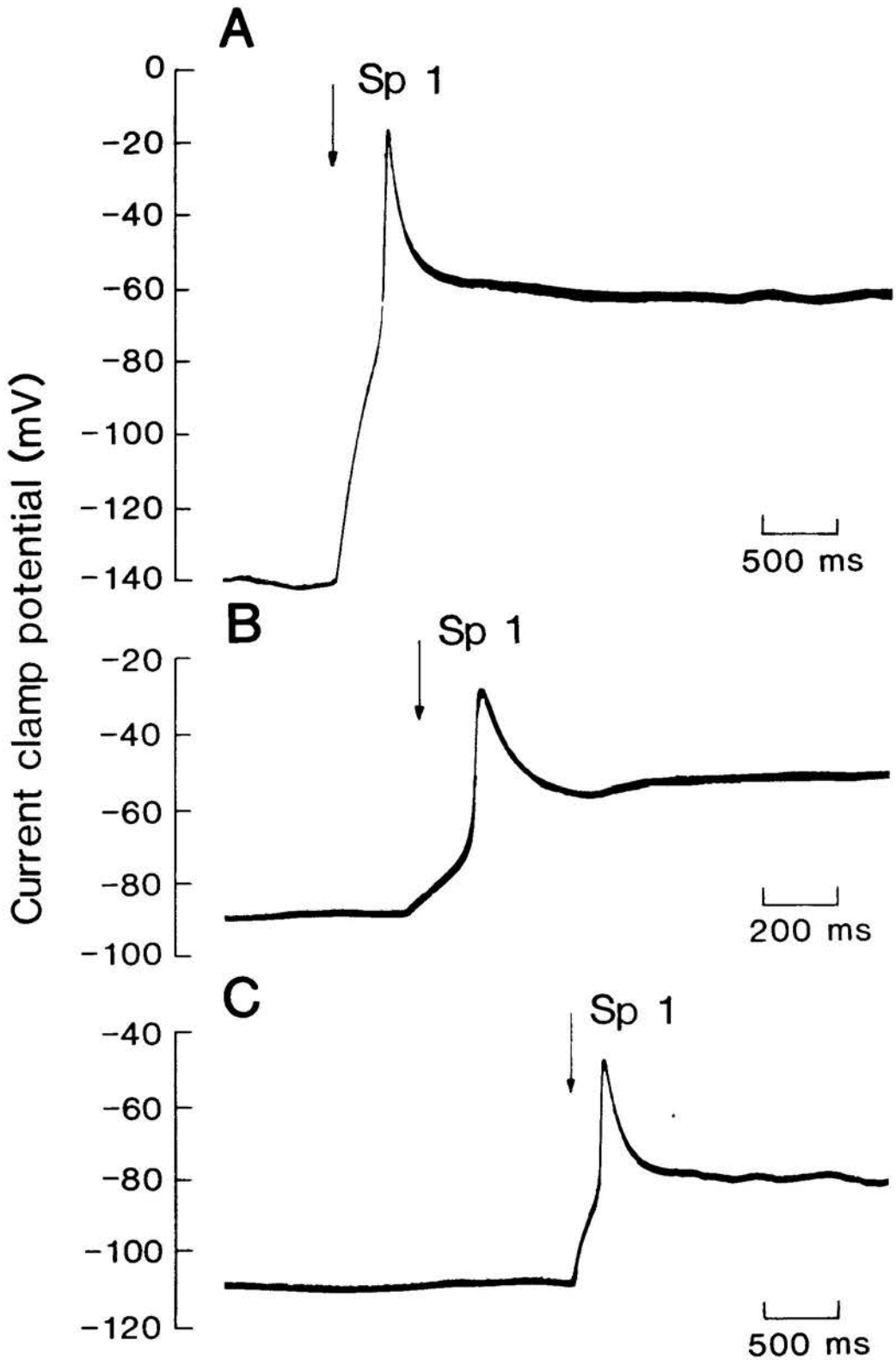
between one and three spontaneous action potentials were recorded but no histological evidence of parthenogenetic activation was found (the duration of such spontaneous action potentials was 18±11 seconds, mean ± SD, n=24). In a further eight eggs in which both spontaneous and sperm evoked action potentials were recorded, fertilization was confirmed by the presence of pronuclei.

In summary, 23 eggs bathed in normal solution gave sperm evoked action potentials after insemination and in seven of those eggs, more than one sperm evoked action potential was observed. In seven eggs fast subthreshold depolarizations (fsds) were observed to accompany sperm fusions. Four eggs which showed sperm evoked action potentials also showed fsds.

In five eggs originally current clamped at high potentials the sperm evoked action potential was followed by a slow depolarization with superimposed trhs. Fig.A.2A shows the sperm evoked action potential observed in one of those experiments and three trhs which followed it (superimposed on a slow depolarization). Fig.A.2Ba is an oscilloscope picture of the sperm evoked action potential shown in Fig.A.2A, and Fig.A.2Bb is a picture of the third trh which followed it (also shown in Fig.A.2A). Figs.A.2Bc and A.2Bd show two trhs which followed a sperm evoked action potential in another egg. It is possible that the slow depolarization occurring after the action potential was caused by a deterioration in the sealing

Figure A.3

Examples of sperm evoked action potentials (oscilloscope pictures) recorded in three different eggs bathed in normal solution.


A
Sp 1
0
-20
-40
-60
-80
-100
-120
-140
500 ms
B
Sp 1
-20
-40
-60
-80
-100
200 ms
C
Sp 1
-40
-60
-80
-100
-120
500 ms
Current clamp potential (mV)

between electrode and egg membrane. Although the exact origin of the slow depolarization observed in those experiments is not known, the results demonstrate fortuitously that sperm egg fusion can cause an action potential and a trh response in the same egg.

The time course of the first sperm evoked action potential (accompanying sperm fusion) is illustrated by oscilloscope pictures of three examples in different eggs (Fig.A.3). The egg was current clamped at -140mV, -90mV and -110mV in Figs.A.3A,B and C respectively. In each case the action potential was triggered by a depolarization whose time course resembled that of electrotonic potentials in hamster eggs (Georgiou et al, 1984). The threshold for the regenerative part of the response to sperm fusion was -80±7 mV (mean ± SD, n=11) which is significantly different to that of electrically evoked action potentials (-60±10mV, mean ± SD, n=8; $P<0.01$, Fisher Behrens test). Sperm evoked action potentials in all but one egg failed to overshoot zero. In thirteen eggs, the peak value of the sperm evoked response was -18±13mV (mean ± SD), whereas electrically evoked responses had a peak value of +3±9 mV (mean ± SD, n=8). In two eggs where the peak value of both responses were measured in the same experiment the electrically evoked action potentials had peak values 30mV and 16mV more positive than their sperm evoked counterparts. The prolonged plateau phase of the sperm evoked response was not observed in the

Figure A.4

A. Oscilloscope picture of an action potential elicited by sperm fusion, in an egg current clamped at -105mV and bathed in Ca2K25. B. Pen trace recording during a fertilization experiment performed in Ca2K25. Lower trace is a continuation of the upper trace. About 10 seconds after sperm addition a sperm fused with the egg (first arrow above the trace), eliciting an action potential which was followed by four brief action potentials (none of these brief action potentials were accompanied by sperm fusion). The latter were followed by another action potential which was caused by a second sperm egg fusion (second arrow above the trace). Histology had confirmed that two sperm had fused with the egg.

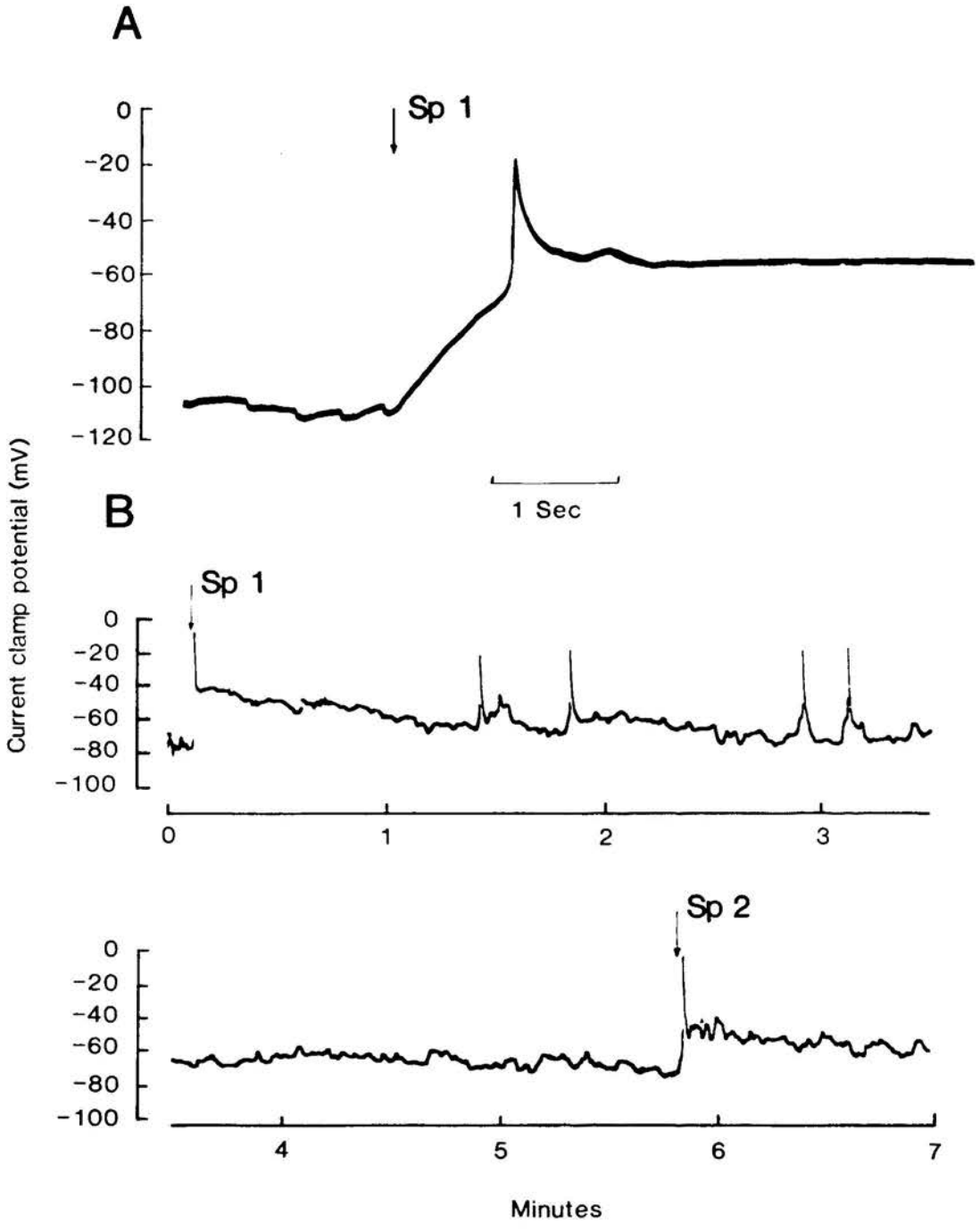

A
Sp 1
0
-20
-40
-60
-80
-100
-120
1 Sec
B
Sp 1
0
-20
-40
-60
-80
-100
0
1
2
3
Sp 2
0
-20
-40
-60
-80
-100
4
5
6
7
Current clamp potential (mV)
Minutes

Table A1

Measurements of various parameters of electrically evoked action potentials, sperm evoked action potentials and fsds, in three different solutions (Ca2K5, Ca4K5 and Ca2K25). The results are shown as mean $\pm$ SD (n=), but on occasions where too few results were available the actual results are quoted (in italics).

SOLUTIONS	ELECTRICALLY EVOKED ACTION POTENTIAL		SPERM EVOKED ACTION POTENTIAL			FSD
	Threshold (mV)	Spike peak (mV)	Threshold (mV)	Spike peak (mV)	Plateau potential level (mV)	Amplitude (mV)
Ca2K5	-60±10 (8)	+3±9(8)	-80±7 (11)	-18±13 (13)	-67±11 (17)	21±11 (10)
Ca4K5		+4±10 (8)				
Ca2K25		+12	-65	-10 -15 -17	-45 -45 -67	

TABLE A1

electrically evoked action potential although a somewhat shorter plateau has been recorded during spontaneous action potentials (Georgiou et al, 1984). In the sperm evoked action potential the plateau potential level was -67±11mV (mean ± SD, n=17).

The amplitude and durations of fsds was found to be 21±11mV (mean ± SD, n=10) and 29±28 seconds (mean ± SD, n=9) respectively.

These results have been summarized in Tables A1 and A2.

A.3 Intracellular recordings during homologous fertilizations of zona-free hamster eggs bathed in high potassium solution current clamped at high potentials

Eight successful fertilization experiments were done in Ca2K25 and the results of two are shown in Fig.A.4. The oscilloscope picture in Fig.A.4A shows that the fusion of a sperm to this egg caused a depolarization which triggered an action potential with a long plateau. Fig.A.4B shows a pen tracing of the current clamp potential from another egg, the lower trace being a continuation of the upper. At zero time sperm were added to the chamber and the first arrow indicates when a single sperm became immotile after attachment to the egg. Four brief action potentials were recorded and then another sperm became immotile while attached to this egg (second arrow).

In summary, eight eggs bathed in high potassium

Table A2

A summary of the durations of spontaneous, brief, and sperm evoked action potentials and the durations of fsds. Results are expressed either as mean ± SD (n=) or the actual values are quoted (in italics).

SOLUTION	DURATION (Seconds)			
	Spontaneous action potentials	Brief action potentials	Sperm evoked action potentials	FSDs
Ca2K5	18±11 (24)	2.4±1.2 (14)	66±78 (15)	29±28 (9)
Ca2K25			12 65 65	

TABLE A2

solution (Ca2K25) gave sperm evoked action potentials and all were confirmed by histology to have been fertilized. Spontaneous action potentials were also recorded in this group of eggs bathed in Ca2K25. None of these was activated except one in which a spontaneous action potential was followed by a sperm evoked action potential.

A.4 Discussion

In this group of experiments many of the responses described in Chapter 5 were also observed, e.g. sperm evoked action potentials, brief action potentials, spontaneous action potentials, fsds and trhs. Although in none of the experiments described in Chapter 5 was a "train" of brief action potentials observed (as in Fig.A.1B and Fig.A.4B.

APPENDIX B STATISTICAL METHODS

B.1 Mean and standard deviation

B.2 Two sample comparisons

a) Two sample t test

b) Fisher Behrens test

c) Wilcoxon signed rank test

B.1 Mean and standard deviation

All the results in this study have been expressed in the form mean $\pm$ SD (n=), both in tables and text. But if the number of values available for any analysis was small, then the actual values have been quoted. For n observations of the variable x (x_1, x_2, ---, x_n) then

$$\text{Mean} = \bar{x} = \frac{\Sigma x}{n}$$

$$\text{SD} = S_x = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

B.2 Two sample comparisons

In order to compare two different samples, one of two types of tests were used. These were the "two sample t test" and "Fisher Behrens test". The former test was used in cases when the ratio of the variance of the two samples was approximately equal to one. For the remaining comparisons the Fisher Behrens test was used. Each of these is discussed more fully below. When it was necessary to compare the medians of two populations, from which observations had been taken in pairs, one from each population then the Wilcoxon signed rank test was used (see below).

a) Two sample t test.

For n observations of the variable x and m of the variable y:-

as in B.1 $\bar{y} = \frac{\sum Y}{m}$

$$S_y = \sqrt{\frac{\sum y^2 - \frac{(\sum y)^2}{m}}{m-1}}$$

The observed t value $= \frac{\bar{x} - \bar{y}}{\sqrt{\frac{(S_x)^2}{n} + \frac{(S_y)^2}{m}}}$

The null hypothesis states that the two population means are equal. This hypothesis was rejected if the observed value of t was greater than the limiting value, given in the conventional t statistic table. The number of degrees of freedom was calculated as (n+m-2).

In this study, the two samples were considered to be significantly different only if the observed t statistic was greater than that in the table, at the 5 per cent probability level.

b) Fisher Behrens test.

If the assumption of equal variances wasnot justified, then the procedure of the preceding section was not followed. For these cases of unequal population variances, the difference of means was assessed by using the Fisher Behrens test.

For this test the observed d was calculated as:-

$$\text{observed } d = \frac{\bar{x} - \bar{y}}{\sqrt{\frac{(S_x)^2}{n} + \frac{(S_y)^2}{m}}}$$

degrees of freedom of x = n-1

degrees of freedom of y = m-1

$$\text{Tan}\,\theta = \frac{(S_x)^2/n}{(S_y)^2/m}$$

Thenull hypothesis of equal population means was rejected if the value of observed d was greater than the tabular value appropriate to the degrees of freedom, θ , and the 5 per cent probability level.

c) Wilcoxon signed rank test.

To perform this test the difference between each pair (one from each population) was calculated. Some differences were positive and some negative (zero differences contribute no information to the comparison and were therefore ignored.) In the next step, the signs of these differences were ignored and the numerical values of the differences were ranked. Then the sum of the ranks of the positive differences was obtained (R+) and similarly those of the negative differences (R-).

The test statistic is the smaller of R+ and R-, denoted by T, the level of significance being associated with it is found from the tables.

APPENDIX C LIST OF ABBREVIATIONS AND DEFINITIONS

AFTER-DEPOLARIZATION-This is a depolarization which sometimes occurred after a trh response. Such a response when recorded with an extracellular pipette has been called an "after-depolarization (I)".

DURATION- The duration of any response, has been defined as the time for it to return to it's initial membrane or it's initial current clamp potential.

FAST RESPONSE-This defines the group of responses observed to accompany sperm fusions. They include fsds and sperm evoked action potentials.

FAST SUBTHRESHOLD DEPOLARIZATION (fsd)-Such a response was observed to accompany some sperm fusions during fertilization experiments with intracellular electrodes. These responses had fast rates of depolarization (compared to trds and trd spikes) and their peak potential was more negative than the threshold of the sperm evoked action potential.

HIGH POTASSIUM- This phrase has been used to describe solutions containing 25mM potassium.

HIGH POTENTIAL- This defines any potential (membrane or current clamp) which is more negative than the threshold for the electrically evoked action potential.

HIGH POTENTIAL EGG - This is an egg in which the recorded membrane potential is more negative than the threshold for the electrically evoked action potential.

LOW POTENTIAL - This defines any potential (membrane or current clamp) which is more positive than

the threshold for the electrically evoked action potential.

LOW POTENTIAL EGG - This is an egg in which the recorded membrane potential is more positive than the threshold for the electrically evoked action potential.

NORMAL POTASSIUM - This describes solutions containing 5mM potassium.

NORMAL SOLUTION - The composition of this solution is given in Chapter 2 and has been abbreviated in the text as Ca2K5 because it contains 2mM calcium and 5mM potassium. This is a solution in which the eggs were stored and isolated.

PEAK POTENTIAL - This is the most depolarized potential of fsds, trds , trd spikes and sperm evoked action potentials (excluding the "spike" in trd spikes and sperm evoked action potentials) and the most hypepolarized potential of trhs.

PLATEAU POTENTIAL LEVEL - This refers to the potential (membrane or current clamp) of the plateau of a sperm evoked action potential.

PREDEPOLARIZATION- This is the name given to a small depolarization occurring immediately before trds, trd spikes, fsds and sperm evoked action potentials which accompany sperm fusions. Such a response when recorded with an extracellular pipette has been called a "predepolarization (I)".

SLOW RESPONSES - This defines a group of responses observed during fertilization of zona-free hamster eggs with hamster sperm. These responses are recurrent and

not always associated with sperm fusion. They have been called slow responses because their rate of change of potential to peak is slower than the other group called fast responses (i.e. fsds and sperm evoked action potentials).

SOLUTIONS - Various types of bathing solutions were used during the experiments. Each solution is described by an abbreviated name. The concentration of calcium, barium, lanthanum, magnesium and strontium is used to form the abbreviated name of any given solution (the chemical symbol of each multivalent ion followed by its concentraton in mM). But if the concentration of any of the above mentioned ions was zero then it was not used to form the abbreviated name. Similarly if the concentration of magnesium was as in the normal solution (i.e. 1.2mM) then it was not used to form the abbreviated name (the concentration of magnesium was 1.2mM in all solutions except the magnesium substituted calcium free solutions). The concentration of potassium in any given solution was also used to form the abbreviated name of that solution. In high potassium solutions, the potassium concentration was elevated by an equimolar replacement of sodium chloride with potassium chloride. In solutions containing a concentration of any of the five above mentioned multivalent ions greater than 5mM, the concentration of sodium chloride was reduced by the appropriate amount to maintain osmolality.

SPIKE PEAK - This refers to the potential at the most depolarized level in trd spikes, electrically evoked action potentials and sperm evoked action potentials.

TRANSIENT RECURRING DEPOLARIZATIONS (trd) - These responses, as the name suggests, are a recurrent phenomenon, observed at varying times after sperm fusion. They are defined as being one of the slow responses, and their initial phase is depolarizing, being only observed at potentials more negative than the potassium equilibrium potential.

TRANSIENT RECURRING HYPERPOLARIZATIONS (trh) - These responses are recurrent, observed after sperm fusion, are slow responses and their initial phase is hyperpolarizing, being only observed at potentials more positive than the potassium equilibrium potential. Such a response when recorded with an extracellular pipette has been called a "trh (I)".

TRH SPIKE - This is the name given to a trh response, superimposed on the repolarizing phase of which is a spike. It only occurs in some eggs fertilized in normal potassium solutions, and that only if 1) the membrane or current clamp potential is more positive than the threshold of the electrically evoked action potential and 2) the peak (i.e. the most hyperpolarized potential) is more negative than the threshold of the electrically evoked action potential. Such a response when recorded with an extracellular pipette has been called a "trh spike (I)".

TRD SPIKE - This essentially is a transient recurring depolarization, upon which is superimposed a spike. Such a response is observed only in high potassium solutions, and that because the potassium reversal potential is more positive than the threshold of the electrically evoked action potential. Therefore if the peak of the trd is more depolarized than the threshold then a trd spike is observed, and not a trd (so long as the membrane or current clamp potential is more negative than the threshold).

VERY HIGH POTASSIUM - This describes solutions containing 40mM potassium. Only a few experiments were performed in such solutions, the results of which are listed in the tables, but not discussed in the text.

APPENDIX D PUBLICATIONS

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CALCIUM-EVOKED OPENING OF POTASSIUM CHANNELS IN HAMSTER EGGS

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SUMMARY

Measurements of membrane potential and resistance have been made in zona-free hamster eggs. The resting potential lay in the range −9 to −100 mV and the input resistance fell in the range 14 to 440 MΩ; high resting potentials were associated with large input resistances. Calcium injected ionophoretically into an egg from an intracellular micro-electrode caused a reduction of the membrane resistance. The estimated reversal potential for the calcium-evoked response was about −80 mV and its amplitude depended on the extracellular concentration of potassium but not on the chloride concentration. We conclude that membrane potassium channels open in response to a rise in the cytosolic concentration of calcium ions. Evidence is presented to suggest that micro-electrode recordings of the membrane potential and resistance of eggs suffer from an impalement leak artifact. The presence of the artifact lowers the resting potential and resistance of the cell so that intracellular calcium injection causes a hyperpolarization. We conclude that a hyperpolarizing response to calcium would be unlikely in the absence of an impalement artifact.

INTRODUCTION

Miyazaki & Igusa (1981, 1982) have reported that the attachment of a hamster sperm to a hamster egg causes a series of transient hyperpolarizations of the egg's plasma membrane. It appears that calcium injection into a hamster egg mimics the effect of sperm attachment on the egg's membrane potential. However, Miyazaki & Igusa (1982) referred to unpublished results showing that calcium injection into hamster eggs produced a fall in membrane conductance whereas they presented evidence that sperm attachment evoked a transient increase in membrane conductance. It is difficult to reconcile these conflicting observations and therefore we decided to re-examine the effect of calcium injection on the membrane conductance of hamster eggs. A preliminary account of the work described in this paper has been given to the Physiological Society (Bland, Bountra, Georgiou & House, 1983).

METHODS

Egg donors

Mature virgin female golden hamsters, maintained under controlled lighting (8 h dark/16 h light), were injected I.P. with 30 i.u. pregnant mare serum gonadotrophin (Folligon; Intervet Labs. Ltd, Cambridge) in the early evening. Forty-eight hours later they were injected I.P. with 45 i.u. HCG (Chorionic Gonadotropin CG-2 Sigma Chemical Co., St Louis, U.S.A.).

The hamsters were killed, the oviducts opened and the eggs removed 15–18 h after the last injection.

Pre-treatment of eggs

All eggs were removed from the oviducts in a solution containing (mM): NaCl, 120; KCl, 5; $CaCl_2$, 4; $MgCl_2$, 1·2; Na lactate, 20; Na pyruvate, 1·0; glucose, 5·6; HEPES, 5; NaOH, 2·5. This medium, referred to as normal solution in this paper, had a pH of 7·3 and also contained bovine serum albumin

(4 mg/ml; Sigma). In some of the initial experiments the normal solution contained $CaCl_2$ at a concentration of 2 mM and was buffered at pH 7·6 with Tris–Tris Cl (Bland *et al.* 1983). The potassium concentration in the normal solution was varied by partial or complete replacement of NaCl by KCl. Similarly, the chloride concentration was changed by partial replacement of NaCl by Na propionate.

To remove the cumulus oophorus each egg was incubated for 2–4 min in normal solution containing Hyaluronidase (1 mg/ml; Type 1-S, Sigma). To remove the zona pellucida each egg, freed from cumulus, was bathed for 1–3 min in normal solution containing Trypsin (1 mg/ml; Type III, Sigma). The enzyme treatments were carried out at room temperature (20–22 °C).

Intracellular recording

An egg was placed in a chamber mounted on an inverted microscope (Biovert; Reichert, Austria). Normal solution at room temperature (20–22 °C) was pumped through the chamber (vol. 5 ml) by a Watson-Marlow H.R. Flow inducer (MRHE 200) at a rate of about 5 ml/min. The chamber contained two solid Ag/AgCl electrodes in contact with the solution.

Two micro-electrodes were inserted into the egg for potential recording and current passage. It was not necessary to immobilize the cell because it gently adhered to the glass base of the chamber. The first electrode (50 MΩ), filled with 2 M potassium acetate, was used for recording the intracellular potential relative to the first bath electrode. This micro-electrode was connected to the input of a high impedance pre-amplifier (Model KS 700; WP Instruments Inc., U.S.A.). Current pulses from a Devices stimulator triggered by a Digitimer (D4030; Devices Ltd) were passed between the barrel of the micro-electrode and the first bath electrode by means of a bridge circuit. The resulting electrotonic potentials were monitored for the determination of the current–voltage relation of the cell. The second micro-electrode (10 MΩ), filled with 1 M-$CaCl_2$, was used for the ionophoretic injection of calcium ions into the egg. Current pulses from a Devices stimulator were passed between one end of a 1 GΩ resistor in series with the barrel of the micro-electrode and the second bath electrode. The charge transfer through the calcium micro-electrode required to evoke the electrical responses described in this paper lay in the range 0·2–5 nC as observed previously by Miyazaki & Igusa (1982).

Permanent experimental records were obtained as pen recorder traces on a Devices M2 Recorder or as photographs of the screen of a storage oscilloscope (RM 5113, Tektronix Ltd).

RESULTS

Electrical properties of eggs

The resting potentials of eggs examined in this study lay in the range -9 to -100 mV, the mean $\pm$ S.D. being -34 ± 16 mV for eighty-five cells. When a rectangular current pulse was passed through the recording micro-electrode an electrotonic potential was recorded. An example of the relation between applied current and voltage response is illustrated in Fig. 1. Evidently the relation between current and voltage is linear in the range 0 to -150 mV as found originally by Miyazaki & Igusa (1982) for hamster eggs. The input resistance was calculated from the linear part of the current–voltage relation; in the example shown in Fig. 1 the resistance was 170 MΩ. Mean $\pm$ S.D. values for the input resistance of eighty-five cells were 147 ± 92 MΩ.

The electrical properties of nineteen eggs were examined in detail (Table 1) to give estimates of their time constants, resistances and capacitances. We measured the diameters of most of these cells so that their apparent surface areas could be estimated; in another set of measurements we found that the mean ($\pm$ S.D.) diameter of fifty cells was 79 (± 3) μm. The specific membrane resistance is about 35,000 $\Omega \cdot cm^2$ and the membrane capacity is 3·0 $\mu F \cdot cm^{-2}$. It is possible that the resistance is underestimated and the capacitance over-estimated (see *Leak pathway*).

A hyperpolarizing current pulse evoked an anode-break response in most cells (Fig. 7*C*) provided that the membrane potential reached about -80 mV during the course of the pulse

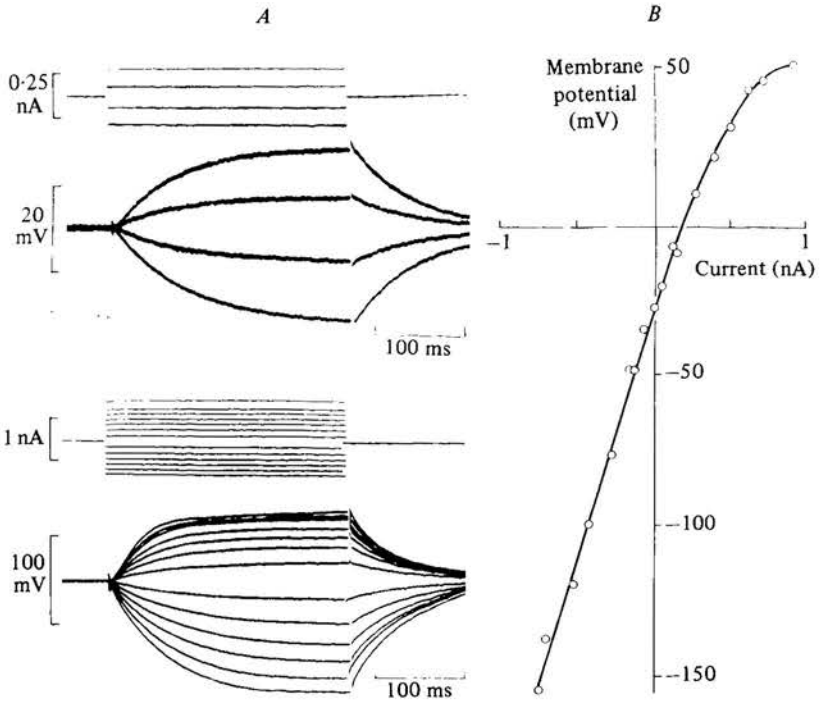

Fig. 1. Current–voltage relation of an egg. *A* shows current pulses passed through the intracellular micro-electrode and the corresponding electrotonic potentials recorded. *B* shows a plot of the relation between the applied current and the membrane potential of the egg.

Table 1. *Electrical properties of eggs*

Egg	Resting potential (mV)	Resistance (MΩ)	Time constant (ms)	Capacity (pF)	Membrane resistance kΩ.cm²	Membrane capacity μF.cm⁻²
1	−23	170	51	290	—	—
2	−44	360	240	660	—	—
3	−46	200	130	650	—	—
4	−48	180	130	720	—	—
5	−27	170	52	310	31	1·7
6	−31	250	63	250	46	1·4
7	−29	54	28	520	10	2·8
8	−28	100	48	460	—	—
9	−32	280	170	610	59	2·8
10	−29	110	76	690	24	3·2
11	−35	170	98	590	—	—
12	−43	130	93	700	—	—
13	−40	130	120	920	29	4·2
14	−28	96	88	920	18	4·9
15	−36	260	160	620	56	2·8
16	−29	100	70	690	—	—
17	−17	78	53	680	—	—
18	−36	180	100	570	35	2·9
19	−44	190	130	650	41	3·0
Mean ± S.D.	−34 ± 8	170 ± 78	100 ± 52	610 ± 180	35 ± 16 ($n = 10$)	3·0 ± 1·0 ($n = 10$)

as observed by Miyazaki & Igusa (1982). This phenomenon has been observed also in mouse eggs and is caused by the opening of voltage-sensitive calcium channels inactivated at the resting potentials usually observed (Okamoto, Takahashi & Yamashita, 1977).

Leak pathway

Continuous monitoring of resting potential and input resistance after an egg was impaled by a micro-electrode revealed that potential and resistance gradually increased over a period of about 10 min (Figs. 3–5). The progressive increases in potential and resistance suggest improved sealing of the micro-electrode to the cell membrane. Even when steady values of both potential and resistance have been reached it is possible that a significant leak impalement artifact remains (Hagiwara & Jaffe, 1979; Petersen, 1980). Miyazaki & Igusa (1982) reported that more negative resting potentials were associated usually with larger input resistances of hamster eggs but they provided no details. We have measured resting potential and input resistance for each cell, a suitable time being allowed for potential and resistance to attain steady values. There is a linear relation between them (Fig. 2). A simple equivalent circuit model with a variable leak resistance can describe the apparent relation in Fig. 2. If a leak pathway exists at the point of insertion of the micro-electrode then the effective input resistance, R', becomes $R_L R/(R_L+R)$ and the potential, E', is given by $(E_L R+ER_L)/(R_L+R)$ where R = cell resistance, R_L = leak pathway resistance, E = cell potential and E_L = leak pathway potential. It can be shown that R' and E' are linearly related by

$$E' = \left(\frac{E-E_L}{R}\right) R' + E_L$$

provided R, E and E_L are constant. According to this model the slope of the linear relation is $(E-E_L)/R$ and the intercept on the potential axis is E_L. Although it is not possible to vary R_L in order to test the hypothesis, the linear relation in Fig. 2 indirectly supports this model since it is expected that R_L will vary from cell to cell. It is also probable, of course, that R, E and E_L will vary from cell to cell so we decided to examine the relation between potential and resistance in individual eggs shortly after impalement. Fig. 3 shows the results of such an experiment. The upper part of the Figure is a pen trace of the membrane potential after micro-electrode insertion at zero time. The lower part is the corresponding graph of resistance and potential, the measurements being made at different times. Evidently there is again, in the case of a single cell, a linear relation between potential and resistance which is compatible with a progressive increase in R_L as the micro-electrode seals to the cell membrane.

A further kind of experimental result suggesting the presence of an impalement leak artifact is illustrated in Fig. 4. The crux of the experiment was to observe the effect of inserting a second micro-electrode (μe 2) on the membrane potential recorded by a previously inserted electrode (μe 1). The intracellular recordings show that the insertion of the second electrode caused a fall in the potential and resistance recorded by the first electrode. Both electrodes recorded the same membrane potential and electrotonic potentials generated by current pulses passed through the first electrode (Fig. 4).

Ionophoretic injection of calcium

We injected calcium ions ionophoretically into a hamster egg by passing a depolarizing current pulse through an intracellular micro-electrode filled with 1 M-$CaCl_2$ to see whether cytosolic calcium influences membrane permeability. The experimental protocol required

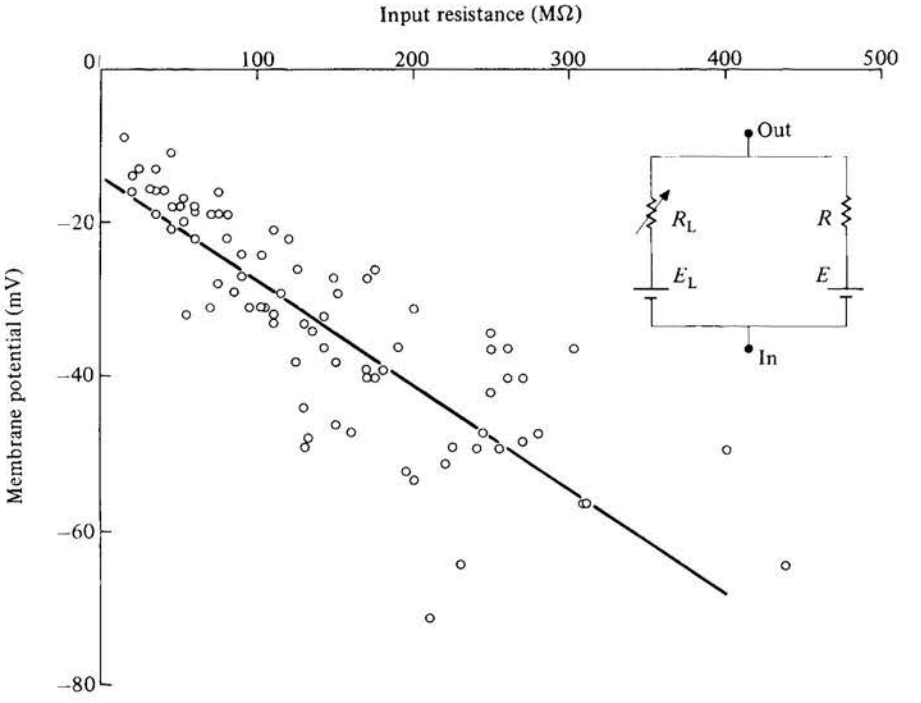

Fig. 2. Relation between estimates of input resistance and membrane potential of eggs. Each point indicates the values for a single egg. The line was obtained by linear regression analysis, the correlation coefficient being −0·7822 for 83 degrees of freedom ($P < 0·001$). Inset Figure is an equivalent circuit model used to account for the relation between resistance and potential (see text). One egg had a resting potential of −100 mV and a resistance of 275 MΩ; this point has not been plotted on the graph although it has been included in the regression analysis.

the insertion of two micro-electrodes, the first for voltage recording and the second for calcium injection. Frequently the insertion of the calcium pipette was not satisfactory because it caused a permanent fall in resting potential and a drop in input resistance (Fig. 5*A*). However, occasionally the calcium pipette could be inserted successfully (Fig. 5*B*). A satisfactory insertion was indicated by a transient hyperpolarization and a transient fall in resistance. Even after a satisfactory impalement by the calcium pipette success was not assured because sometimes the pipette failed to pass sufficient current or developed a poor current-passing property during the experiment.

In forty-five successful experiments a depolarizing current pulse through the calcium pipette was followed by a hyperpolarization lasting 10–30 s. The amplitude and duration of the hyperpolarization evoked by ionophoretic injection of calcium were graded with the quantity of charge ejected from the calcium pipette (Fig. 6). Responses to large ionophoretic pulses of calcium were prolonged and their amplitudes were reduced by raising the concentration of potassium in the bathing fluid (Fig. 6).

In control experiments where the calcium pipette was replaced by a similar one filled with 2 M potassium acetate it was found that depolarizing currents did not elicit hyperpolarizing responses (Fig. 7*A*). In some cases (not illustrated) a small depolarizing response followed the pulse possibly because of temporary resistive breakdown of the membrane.

By contrast, a hyperpolarizing current pulse through the control pipette or a calcium pipette occasionally produced a small transient hyperpolarization (Fig. 7*A*, *B*).

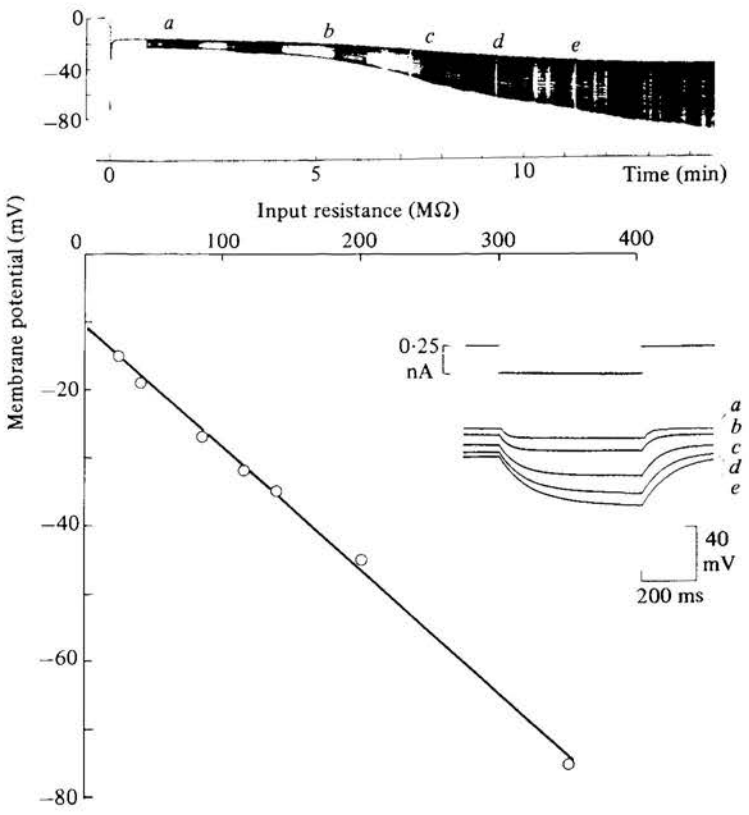

Fig. 3. Progressive increase in the membrane potential and the input resistance of an egg after impalement by a micro-electrode. The upper part is a pen tracing showing the initial period of recording after impalement, the downward deflexions being produced by constant current pulses passed through the recording micro-electrode. Below is a plot of the membrane potential against the input resistance measured at times (*a–e*) during the recording. The potential and resistance continued to increase (not shown) to maximum values of −75 mV and 350 MΩ respectively. Oscilloscope pictures of current pulses and electrotonic potentials recorded at *a*, *b*, *c*, *d* and *e* are superimposed beside the graph. The line was obtained by linear regression analysis, the correlation coefficient being −0·9990 for 5 degrees of freedom ($P < 0·001$).

The small responses to hyperpolarizing current pulses passed through the calcium or control pipette probably are caused by the calcium influx responsible for the anode-break response recorded in hamster eggs (Fig. 7*C*) (Miyazaki & Igusa, 1982). We have found that anode-break responses are blocked by the presence of cobalt or lanthanum ions in the bathing solution.

To monitor the change in membrane conductance produced by calcium injection we passed constant current pulses through the recording micro-electrode. Because the current–voltage relation of the hamster egg is linear in the range 0 to −150 mV hyperpolarizing current pulses were applied. Calcium injection caused a rise in membrane conductance as judged by the reduction of the electrotonic potentials which are proportional to membrane resistance (Fig. 8). Generally the fall in resistance outlasted the hyperpolarization.

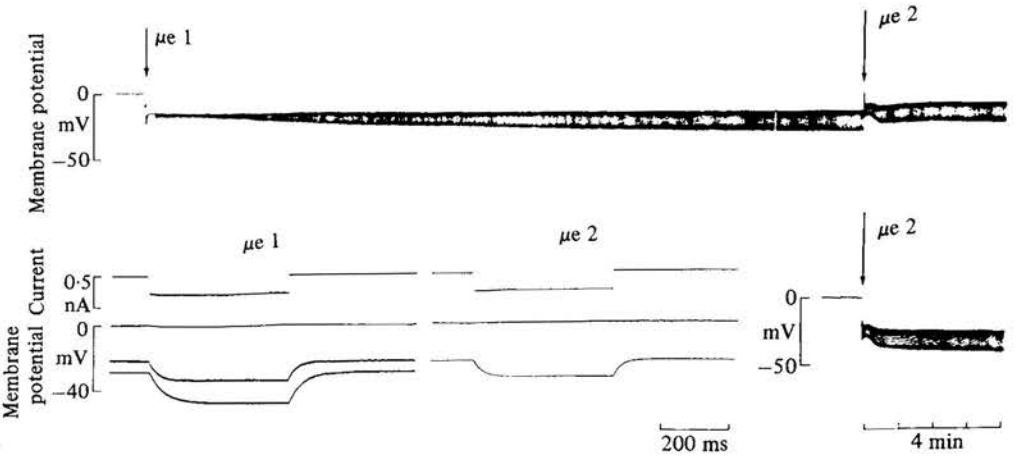

Fig. 4. Insertion of two micro-electrodes into a hamster egg. The upper pen trace shows the membrane potential recorded by the first micro-electrode (μe 1) before and after insertion of the second micro-electrode (μe 2). The downward deflexions were produced by constant current pulses passed through the first micro-electrode. The lower pen trace shows the membrane potential recorded by the second micro-electrode, the downward deflexions being caused by the current pulses passed through the first micro-electrode. Alongside the lower pen trace are oscilloscope pictures of current pulses and electrotonic potentials recorded by both micro-electrodes just after insertion of the second micro-electrode. The lower oscilloscope trace recorded by the first micro-electrode shows the electrotonic potential recorded just before the insertion of the second micro-electrode.

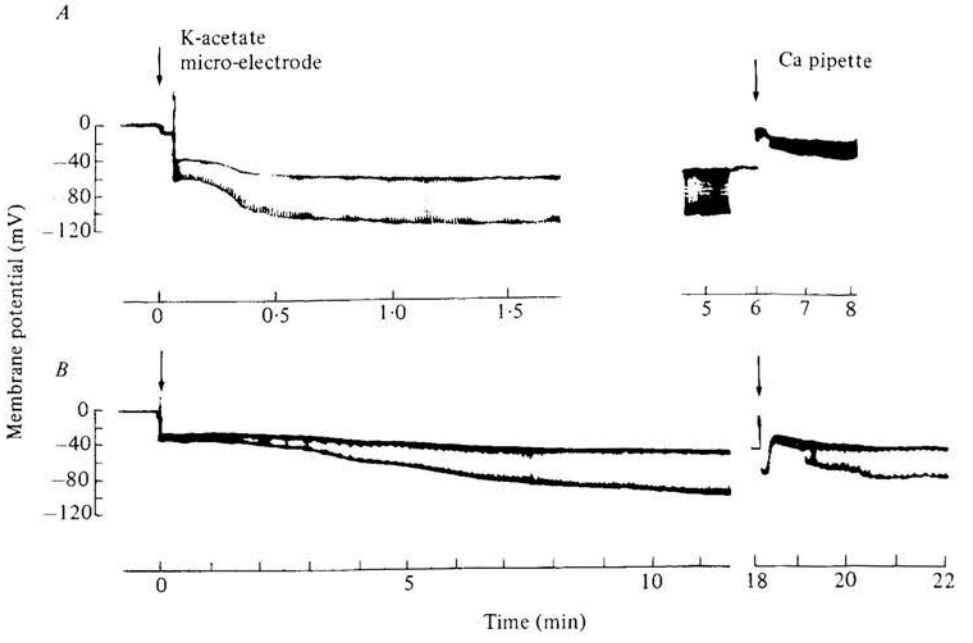

Fig. 5. Insertion of two micro-electrodes into an egg, one being filled with potassium acetate and the other with calcium chloride. The downward deflexions were produced by constant current pulses passed through the potassium acetate micro-electrode and the arrows indicate the times at which attempts were made to insert the electrodes. *A* shows the trace of the membrane potential recorded by the potassium acetate electrode. At the second arrow the attempt to insert the calcium pipette failed since there was a fall in both membrane potential and resistance. *B* shows the results of another experiment in which the attempt to insert the calcium pipette (second arrow) was successful since the changes in potential and resistance were transient only (full recovery not shown).

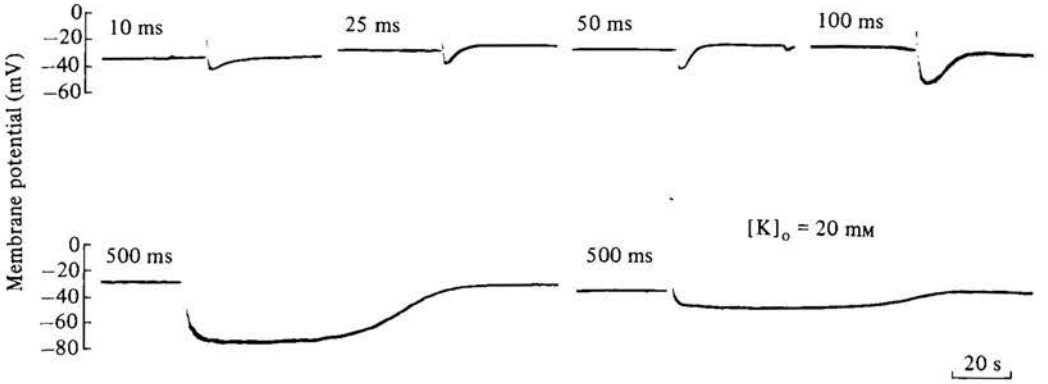

Fig. 6. Effect of the duration of the current pulse passed through a calcium pipette on the membrane potential of an egg. The pulse durations are given above each section of the trace showing responses to injected calcium. A maximum response was obtained with a pulse duration of 500 ms and this response was reduced when the extracellular concentration of potassium was increased from 5 to 20 mM.

Analysis of records

As indicated by Trautwein & Dudel (1958), values for the conductance, g and the reversal potential, e, at the peak of a response may be derived from a single record of the kind shown in Fig. 8. Relations equivalent to those of Trautwein and Dudel are derived here as follows. Evidently for a current pulse I giving electrotonic potentials P and p at rest and during the response respectively we have

$$P = \frac{I}{G} \quad \text{and} \quad p = \frac{I}{G+g},$$

where G is the resting conductance. For a resting potential E and a response v we find

$$g = \left(\frac{P-p}{p}\right) G$$

and

$$e = \left(\frac{P}{P-p}\right) v + E.$$

These relations would not be valid unless the resting conductance were independent of the current and hence of the membrane potential as we have found (Fig. 1).

To illustrate the method of analysis we consider the examples shown in Fig. 8. The intracellular recording in Fig. 8 (upper) shows $E = -19$ mV, $v = -30$ mV, $P = -20$ mV and $p = -10$ mV and these yield $e = -79$ mV. The corresponding values in Fig. 8 (middle) are $E = -35$ mV, $v = -29$ mV, $P = -87$ mV and $p = -42$ mV and hence $e = -91$ mV. By this method we determined the reversal potentials for calcium-evoked responses in thirty-three cells (Table 2).

During the course of experiments we noticed that spontaneous hyperpolarizations occurred in some eggs. Fig. 9 shows two striking examples of large spontaneous hyperpolarizations. Applying the analysis outlined above we have estimated the reversal potentials for these spontaneous events to be -80 and -90 mV. These values fall within the range observed in the calcium injection experiments. The mean value of the reversal potential for

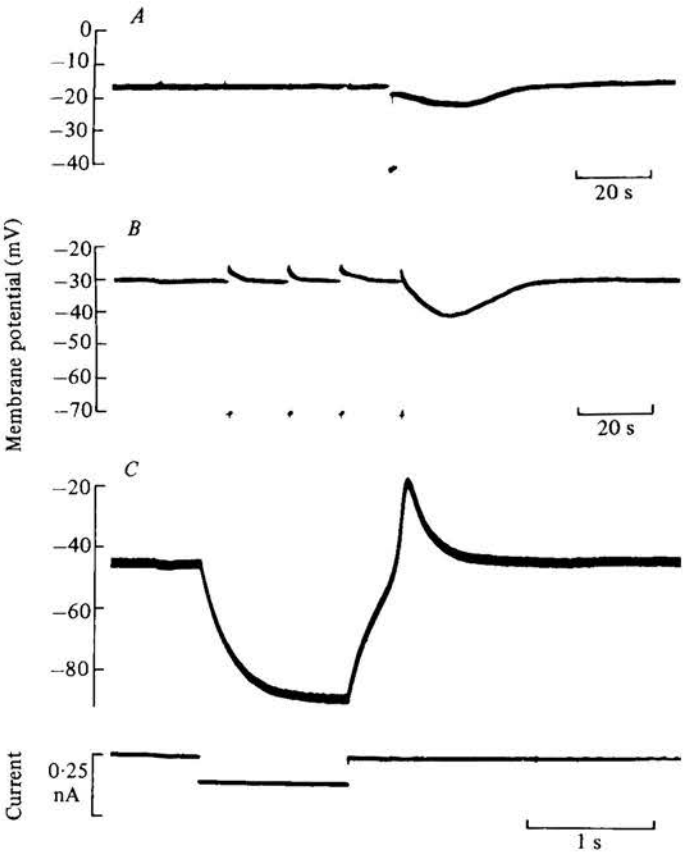

Fig. 7. Effect of current passage through micro-electrodes on the membrane potential of eggs. *A* shows the effect of large current pulses passed through a low resistance micro-electrode filled with 2 M potassium acetate. The first four pulses were depolarizing and had durations of 100, 250, 500 and 1000 ms respectively; the fifth pulse was equal but of opposite polarity to the preceding pulse. *B* shows the effect of large hyperpolarizing current pulses passed through a calcium pipette on the membrane potential recorded by a potassium acetate micro-electrode inserted into the same cell. *C* shows the effect of a hyperpolarizing current pulse (lower trace) passed through a potassium acetate micro-electrode on the membrane potential (upper trace) recorded by the same electrode. Note the anode-break response at the end of the hyperpolarizing electrotonic potential.

the response to calcium is −79 mV and this suggests that calcium causes an increase in membrane permeability to chloride or potassium ions.

Ionic basis of response to calcium

We injected calcium into four eggs bathed in a solution containing a chloride concentration of 10 mM. This solution caused depolarization of each egg and the mean resting potential fell from −38 to −24 mV. Calcium injection caused a hyperpolarizing response with a mean reversal potential of −74 mV. Thus the potential change produced by a rise in cytosolic calcium concentration does not depend on the opening of chloride channels.

The resting potential of hamster eggs is relatively insensitive to the potassium concentration in the bathing solution (Miyazaki & Igusa, 1982) as we have confirmed in this study. In

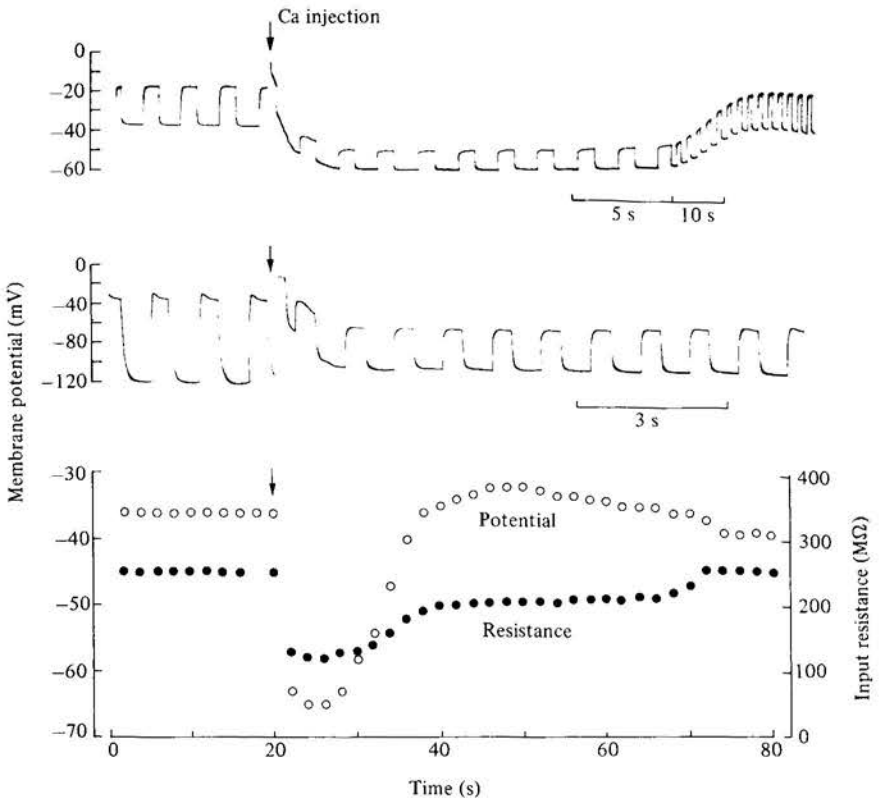

Fig. 8. Effect of calcium ionophoretically injected into eggs on their membrane potential and resistance. Arrows indicate the time at which calcium was injected ionophoretically into the eggs. Upper and middle traces show results of two experiments on different eggs. The hyperpolarizing electrotonic potentials were produced by constant current pulses passed through the recording micro-electrodes. The graph displays the results of the entire response to calcium which is illustrated partially by the middle trace.

Table 2. *Reversal potential* for response to injected calcium*

Resting potential (mV)	Input resistance (MΩ)	Reversal potential (mV)	Response to calcium	
			Hyperpolarization (mV)	Resistance† (MΩ)
-37 ± 14	160 ± 73	-79 ± 13	23 ± 9	50 ± 30

* Mean ± S.D. values (thirty-three cells).
† Minimum value.

each of eight eggs it was possible to determine the reversal potential for the response to calcium injection at two different concentrations of potassium. An example is illustrated in Fig. 10 which shows the electrotonic potentials produced by constant current pulses under resting conditions and during responses to calcium when the cell was bathed in solutions containing 1 or 125 mM potassium. The reversal potential when $[K]_o = 1$ mM was -98 mV and when $[K]_o$ was changed to 125 mM it became -13 mV. The shift of 85 mV is less than

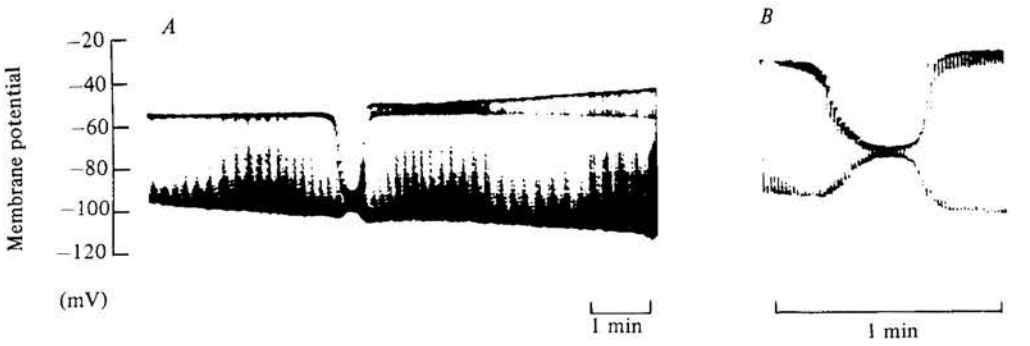

Fig. 9. Examples of spontaneous hyperpolarizations recorded from eggs. The brief downward deflexions in *A* and *B* were produced by constant current pulses passed through the recording micro-electrode.

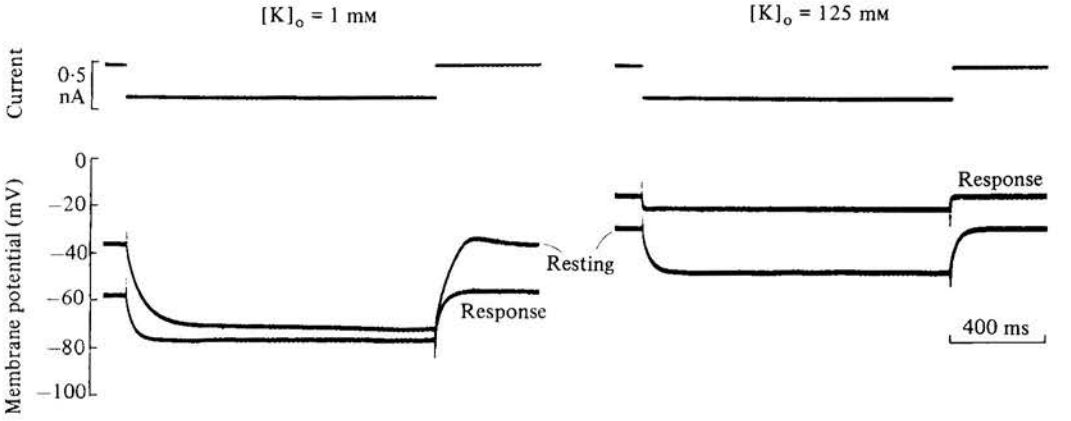

Fig. 10. Effect of the extracellular concentration on the electrical response to calcium injection into an egg. Upper and lower traces are superimposed traces of current pulses and electrotonic potentials respectively. When $[K]_o$ = 1 mM the response to calcium was a hyperpolarization whereas when $[K]_o$ = 125 mM the response was a depolarization. In both conditions the amplitudes of the electrotonic potentials were reduced.

the theoretical value of 122 mV according to Nernst. In contrast, the corresponding shift of the resting potential was from −42 to −33 mV. The mean slope of the relation between the reversal potential and log $[K]_o$ for the results shown in Fig. 11 is 50 mV whereas that expected according to Nernst is 58 mV.

Values of the reversal potential have been determined at external concentrations of potassium of 1, 5, 40 and 125 mM (Fig. 11). The strong dependence of the reversal potential on potassium concentration indicates that calcium injection opens potassium channels in the plasma membrane of hamster eggs.

An indirect finding which strongly supports the idea that cytosolic calcium controls the potassium permeability of hamster eggs is shown in Fig. 12. In this experimental record, which is representative of several others, a single intracellular electrode monitored changes in the resting potential which occurred when hyperpolarizing current pulses were passed through it. Trains of hyperpolarizing current pulses caused hyperpolarizing responses when $[K]_o$ = 1 or 5 mM and a depolarizing response when $[K]_o$ = 125 mM. We interpret this record to mean that the anode-break responses evoked by hyperpolarizing pulses produce a sufficient rise in cytosolic calcium concentration to open membrane potassium channels.

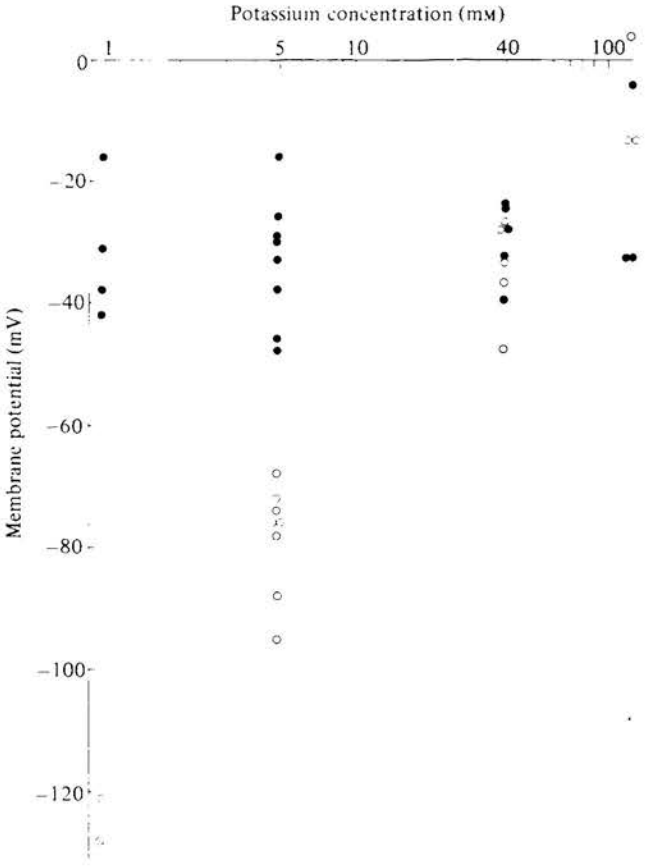

Fig. 11. Effect of the extracellular concentration of potassium on the resting potential (●) and the estimated reversal potential (○) in the same cell during the response to injected calcium.

In separate experiments we found that the hyperpolarizing response of an egg to a hyperpolarizing current pulse passed through the calcium pipette was reversibly abolished by the presence of 1 mM lanthanum nitrate in the bathing solution whereas the hyperpolarizing response to injected calcium was not abolished by lanthanum ions.

DISCUSSION

The main object of our experiments was to establish whether or not ionophoretic injection of calcium ions into hamster eggs altered plasma membrane resistance.

We have shown that plasma membrane potassium channels in the hamster egg are opened directly by ionophoretic calcium injection or indirectly by calcium influx by electrical excitation of the membrane. A rise in cytosolic calcium concentration produces an observable increase in membrane conductance which underlies a hyperpolarizing response with a potassium-dependent reversal potential as found for the sperm-evoked response in hamster eggs by Miyazaki & Igusa (1982). Such a calcium-mediated increase in membrane potassium permeability has been found in numerous cells including erythrocytes (Gardos, 1958), neurones (Meech, 1978) and gland cells (Petersen, 1980).

In common with previous findings the time course of the calcium-evoked electrical

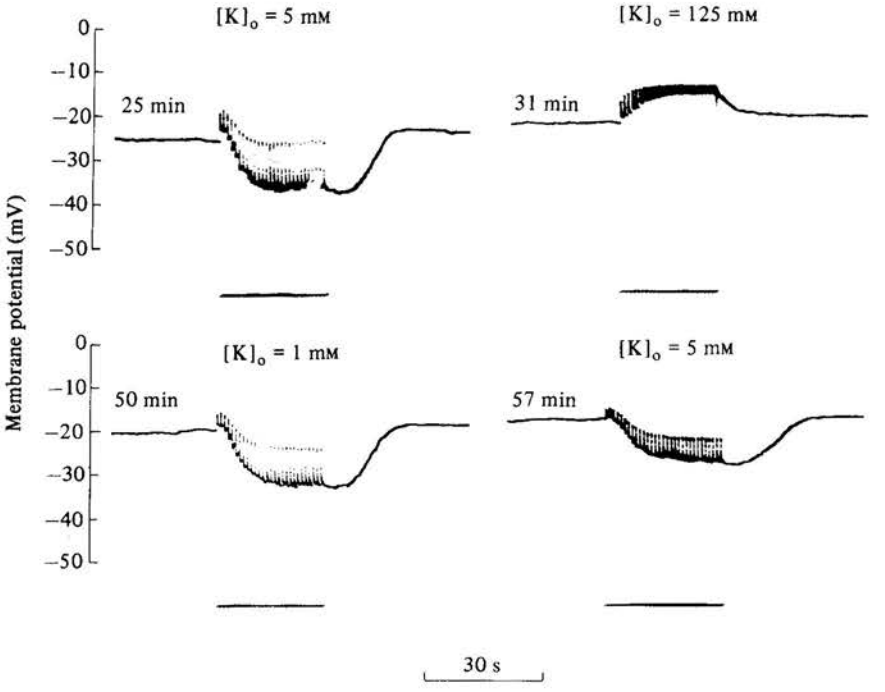

Fig. 12. Responses of an egg bathed in different potassium concentrations to trains of hyperpolarizing current pulses passed through the recording micro-electrode. The times after impalement at which the current pulses were delivered, are given above each extract from the continuous record of the membrane potential. The horizontal bars underneath each trace were generated by the pen hitting the physical end of its travel in the recorder.

response is relatively slow in hamster eggs. For example, the time-to-peak, t_p, is about 3 s. On the assumption that the tip of the calcium pipette acts as a point source during the ionophoretic pulse then $t_p = x^2/6D$ where x is the diffusion pathlength between the pipette tip and the membrane and D is the effective diffusion coefficient (see del Castillo & Katz, 1955). The value of x must be less than the cell's radius, i.e. 40 μm. Hence on a diffusion model of the response to calcium D must be smaller than 10^{-6} cm^2.s^{-1} which is plausible in view of its slow diffusion in aqueous solution and of the calcium buffering power of cytoplasm (cf. Hodgkin & Keynes, 1957).

Although the results presented in this paper suggest that calcium injection can mimic the electrical effect of sperm attachment to hamster eggs it is not known how sperm attachment might produce the necessary increase in cytosolic calcium concentration (Miyazaki & Igusa, 1982).

Our electrical measurements with intracellular micro-electrodes point to the existence of a leak pathway at the electrode tip. This leak pathway is sufficient to cause depolarization of the egg. It is our view that previously published values of the potential and resistance of mammalian eggs (Powers & Tupper, 1974; Okamoto *et al.* 1977; Miyazaki & Igusa, 1982) are probably underestimates of the real values. In conditions where the hamster egg is depolarized by the leak impalement artifact the calcium-evoked opening of potassium channels produces a hyperpolarization. It is possible that the real potential of the egg is close to or even exceeds the equilibrium potential for potassium and therefore probable that potassium channel opening causes no change of potential or depolarization.

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Note added in proof. While this paper was in press Igusa & Miyazaki (1983) reported that calcium injection into hamster eggs causes a rise in membrane conductance as described here.

In recent experiments we have found that hamster and mouse eggs with high membrane potentials (−90 mV) and resistances (500 MΩ) show overshooting action potentials in response to depolarizing current pulses.

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CALCIUM ACTION POTENTIALS IN UNFERTILIZED EGGS OF MICE AND HAMSTERS

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SUMMARY

Measurements of membrane potential and resistance have been made in zona-free eggs of mice and hamsters. The mean ± S.D. values for membrane potential were −91 ± 28 mV (mouse) and −97 ± 29 mV (hamster) and for input resistance were 430 ± 230 MΩ (mouse) and 410 ± 150 MΩ (hamster) respectively. Large fluctuations (20 mV) of membrane potential occurred apparently at random and these were accompanied by changes of membrane resistance. Depolarizing current pulses passed through the recording micro-electrode evoked action potentials in eggs of both species. The threshold for excitation was about −50 mV, the maximum rate of rise of the action potential was about 3 $V.s^{-1}$ and its peak value was about +13 mV. Action potentials could be evoked in eggs bathed in sodium-free solution or in normal solution containing tetrodotoxin (3 μM). The presence of cobalt (5–20 mM), lanthanum (1 mM) or verapamil (200–400 μM) in the bathing solution suppressed the action potential. Raising the extracellular calcium concentration from 4 to 40 mM increased the peak value of the action potential by 25 mV. It is concluded that the plasma membranes of mouse and hamster eggs have voltage-dependent calcium channels.

INTRODUCTION

Action potentials have been recorded from the eggs of starfish (Miyazaki, Ohmori & Sasaki, 1975) and sea urchins (Jaffe, 1976; Okamoto, Takahashi & Yamashita, 1977). These regenerative responses arise from the opening of voltage-dependent calcium channels. The possible role of action potentials in the fertilization of invertebrate eggs has been discussed by Hagiwara & Jaffe (1979) and Whitaker & Steinhardt (1982). By comparison, electrophysiological studies of mammalian eggs are not so extensive. It is known, however, that the eggs of hamsters and mice give anode-break responses to hyperpolarizing current pulses (Okamoto *et al.* 1977; Miyazaki & Igusa, 1982). The characteristics of the anode-break responses suggest that they are caused by the opening of calcium channels apparently inactivated in the resting state. Evidence from voltage-clamp experiments on mouse eggs (Okamoto *et al.* 1977) supports the idea that there are voltage-sensitive calcium channels which are inactivated at the resting potential usually observed in these eggs. Recently, however, it has been shown by Georgiou, Bountra, Bland & House (1983) that the membrane potential and input resistance of hamster eggs are substantially larger than previously recorded. It is probable that the earlier measurements of potential and resistance in mammalian eggs suffered from impalement leak artifacts as was the case for intracellular recordings from sea urchin eggs (Hagiwara & Jaffe, 1979; Whitaker & Steinhardt, 1982).

In this paper we report that mouse eggs, like hamster eggs, have large membrane potentials and input resistances and that these mammalian eggs have calcium action potentials which can be evoked by depolarization.

A preliminary account of some aspects of this work has been given to the Physiological Society (Bland, Bountra, Georgiou & House, 1983).

METHODS

Egg donors

Mature female mice (Balb/c strain) and golden hamsters were egg donors in this study.

Mice. Animals were maintained under a fixed light/dark cycle (16 h light/8 h dark). Super-ovulation was induced by injecting (I.P.) 10 i.u. pregnant mare serum gonadotrophin (PMSG) (Folligon, Intervet Labs. Ltd, Cambridge) in the early evening followed by injection (I.P.) of 10 i.u. HCG (Chorionic Gonadotrophin, CG-2 Sigma Chemical Co., St Louis, U.S.A.) forty-eight hours later. Animals were killed 15–18 h after the second injection and their oviducts were placed in a dish containing a physiological solution (called normal) at room temperature (20–22 °C). The compositions of the normal and test solutions are given in Table 1; solutions contained HEPES as a buffer. In some experiments a bicarbonate–CO_2-buffered solution (Table 1) was used and no difference in the electrical behaviour of mouse and hamster eggs was found. Eggs were removed from the oviducts and placed in normal solution containing hyaluronidase (1 mg.ml^{-1}, Type 1-S, Sigma) for 5 min to remove the cumulus oophorus. Removal of the zona pellucida was achieved by transferring eggs (freed from cumulus) into a normal solution containing protease (1 mg.ml^{-1}, Type XIV, Sigma) for 10–15 min at room temperature.

Hamsters. Details of obtaining zona-free eggs from hamsters were similar to those described for mice, with the following exceptions. Animals were injected (I.P.) with 30 i.u. PMSG in the early evening and with 45 i.u. HCG 48 h later. About 16 h after the HCG injection animals were killed, the oviducts opened and eggs transferred into normal solution. To remove the cumulus each egg was incubated for 2–4 min in normal solution containing hyaluronidase (1 mg.ml^{-1}). To remove the zona pellucida each egg (freed from cumulus) was bathed for 1–3 min in normal solution containing trypsin (1 mg.ml^{-1}, Type III, Sigma). Enzyme treatments were carried out at room temperature (20–22 °C).

Intracellular recording

An egg was transferred to a chamber (volume 5 ml) mounted on an inverted microscope (Biovert, Reichert, Austria). Solution was pumped through the chamber by a Watson-Marlow HR flow inducer (MRHE 200) at a rate of about 5 ml.min^{-1}. The chamber contained a solid silver/silver chloride electrode in contact with the bathing solution. In experiments where the chloride concentration of the bathing solution was changed the bath was earthed via a 3 M-KCl saline–agar bridge.

A micro-electrode (30–80 MΩ) filled with 2 M potassium acetate was inserted into the egg for simultaneous potential recording and current passage. It was connected to the input of a high-impedance pre-amplifier (Model KS700, WP Instruments, U.S.A.). Current pulses from a Devices stimulator triggered by a Digitimer (D4030, Devices Ltd) were passed via a bridge circuit between the barrel of the micro-electrode and the bath electrode. Cell impalement was achieved by resting the micro-elecrode on the surface of the egg and increasing the negative capacity applied to the micro-electrode so that it went briefly into electrical oscillation.

RESULTS

All of the experiments described here were made on zona-free eggs of mice and hamsters. In separate experiments on eggs with intact zona pellucida similar results were found so the results presented below are not artifacts caused by the enzymic removal of the zona.

When a micro-electrode was inserted into a mouse egg the recorded membrane potential was initially small (−10 to −30 mV) and then began to increase with time. In about half of the cells examined the potential attained values of about −35 mV and the input resistance reached about 150 MΩ. For reasons which will be discussed later we consider that these cells were damaged by micro-electrode penetration and therefore they have been excluded from the present results. In the remainder of cells examined both the potential and the resistance increased together over a period ranging from 3 to 30 min to reach maximal values around −90 mV and 500 MΩ respectively. Similar progressive increases in potential and

Table 1. *Composition of solutions*

Solution	Na	K	Ca	Mg	Choline	Cl	Lactate	Pyruvate	Glucose	Sucrose
Normal	143·6	5	4	1·2	—	135·4	20	1·1	5	—
Sodium free	—	5	4	1·2	120	132·9	—	—	5	40
High calcium sodium free	—	5	40	1·2	40	124·9	—	—	5	90
High magnesium sodium free	—	5	4	40	40	130·5	—	—	5	80
Low chloride†	143·6	5	4	1·2	—	15·4	20	1·1	5	—
CO_2–bicarbonate*	146·1	5	4	1·2	—	115·4	20	1·1	5	—

Concentrations expressed as mM. All solutions except CO_2–bicarbonate solution contained 5 mM HEPES plus 2·5 mM-NaOH (or KOH) to give pH 7·2.

* The pH of the CO_2–bicarbonate solution was 7·4. This solution contained $NaHCO_3$ at a concentration of 25 mM.

† In most experiments sodium chloride was replaced by sodium methylsulphate; in others chloride was replaced by gluconate or propionate.

resistance after impalement have been observed in the eggs of starfish (Miyazaki *et al.* 1975), sea urchins (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979) and the hamster (Georgiou *et al.* 1983). These authors have attributed the increases in potential and resistance to an improvement of the sealing of the micro-electrode to the cell membrane.

Fig. 1 *A* displays an illustrative example of an intracellular recording from a mouse egg. The pen trace (top) shows the increase in membrane potential with time. Shortly after impalement hyperpolarizing current pulses were passed through the micro-electrode for the period indicated by the horizontal bar. Electrotonic potentials appeared as downward deflexions and progressively increased in size. Over a range of membrane potential each was followed by an anode-break response which gave an upward deflexion. Eventually a membrane potential was reached at which anode-break responses failed to appear and thereafter the membrane potential and the electrotonic potential continued to increase. After about 3 min the current pulses were switched off and the membrane potential continued to climb to about −120 mV and showed spontaneous fluctuations of about 20 mV in amplitude. Brief depolarizing current pulses produced action potentials (●) and oscilloscope pictures of these responses are shown in Fig. 1 *Ba* below the pen trace. Occasionally spontaneous action potentials (marked *, Fig. 1 *A*) were recorded. It is possible that the progressive increase in potential and resistance observed within 5 min of impalement is caused by enhanced sealing of the micro-electrode to the cell. To test this idea a second micro-electrode was inserted into the egg during the passage of hyperpolarizing current pulses (horizontal bar) through the first electrode. Just before the second impalement the input resistance of the cell was 520 MΩ, indicated by the size of the electrotonic potential (lower oscilloscope trace, Fig. 1 *Bb*). Upon the second impalement the potential and the resistance fell transiently; the upper oscilloscope trace in Fig. 1 *Bb* shows the smaller electrotonic potential (accompanied by an anode-break response) shortly after the second impalement. Thereafter the membrane potential and the electrotonic potential recovered their high values and the spontaneous fluctuations of potential reappeared (Fig. 1 *Bc*).

The pen trace and oscilloscope pictures in Fig. 1 show several new features about the electrical behaviour of unfertilized mouse eggs, namely (a) high membrane potential and resistance, (b) large fluctuations of the potential 'at rest', (c) action potentials evoked by depolarization, and (d) spontaneous action potentials. These features, which we have recorded also in unfertilized hamster eggs, will now be discussed in detail.

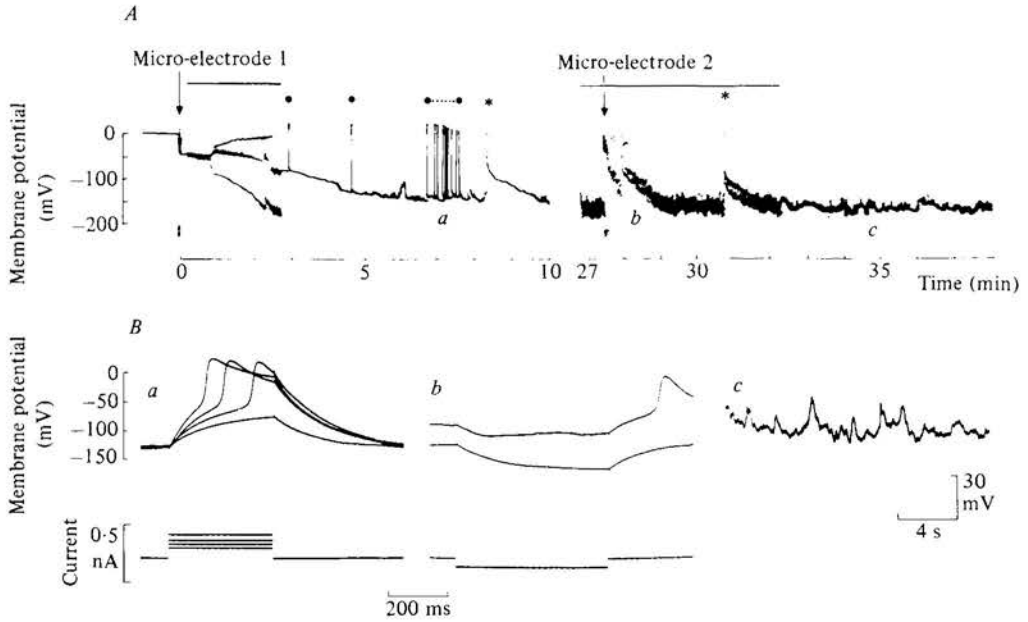

Fig. 1. An intracellular recording from a mouse egg. *A*, pen trace of membrane potential recorded by a micro-electrode (1) after its insertion. Horizontal bars indicate periods when hyperpolarizing current pulses (500 ms) were passed through micro-electrode 1. Brief depolarizing current pulses (●) evoked action potentials. Occasionally spontaneous action potentials (*) were recorded. A second micro-electrode (2) was inserted into the egg about 27 min after the insertion of micro-electrode 1. *B*, oscilloscope pictures of action potentials (*a*), electrotonic potentials (*b*) and spontaneous fluctuations of potential (*c*) recorded at various times after impalement. The times are indicated by *a*, *b* and *c* on the pen trace in *A* above.

Passive electrical properties

Membrane potential, resistance and capacitance

The current/voltage relations of twenty-two mouse eggs were obtained after allowing an appropriate period for the potential and resistance to attain their maximal values. Because the membrane potential fluctuates we estimated the average value from pen recordings for each cell. Table 2 shows the average membrane potential, resistance, time constant and capacitance for each egg. The mean values ($\pm$S.D.) of the membrane potential, resistance and capacitance were -91 ± 28 mV, 430 ± 230 MΩ and 280 ± 110 pF. Hamster eggs also had large membrane potentials and resistances (Table 2). In separate experiments on mouse eggs we measured the diameters of fifteen eggs and found a mean of 80 μm which is close to that reported for hamster eggs (Georgiou *et al.* 1983). On the assumption that the egg's surface equals the apparent area the specific membrane resistance and capacitance of mouse eggs were found to be 86 kΩ . cm^2 and 1·4 μF . cm^{-2} respectively. The values for hamster eggs were 82 kΩ . cm^2 and 3·3 μF . cm^{-2}. If the difference between the membrane capacitances of hamster and mouse eggs reflects a difference in their membrane areas (in spite of their similar diameters) then the above value of the membrane resistance of hamster eggs is an underestimate.

Effect of ionic substitution

When the sodium in the normal solution was replaced by choline the membrane potential increased. This suggests that the relative sodium permeability of the mouse egg is not

Table 2. *Electrical properties of eggs*

Egg	Average membrane potential (mV)	Resistance (MΩ)	Time constant (ms)	Capacitance (pF)
		Mouse eggs		
1	−106	240	100	420
2	−88	470	110	230
3	−63	530	120	220
4	−115	500	92	180
5	−130	660	130	200
6	−109	450	110	240
7	−56	800	170	210
8	−77	520	160	310
9	−100	1100	250	230
10	−54	250	85	340
11	−98	300	65	220
12	−104	310	39	130
13	−130	520	130	250
14	−60	570	65	110
15	−88	450	130	290
16	−73	270	59	220
17	−95	330	150	460
18	−165	400	110	270
19	−75	130	38	290
20	−78	260	69	270
21	−76	240	92	390
22	−59	110	69	600
Mean ± S.D.	−91 ± 28	430 ± 230	110 ± 50	280 ± 110
		Hamster eggs		
1	−95	430	210	490
2	−150	520	220	420
3	−71	620	390	630
4	−85	350	350	1000
5	−75	340	250	740
6	−107	180	120	670
Mean ± S.D.	−97 ± 29	410 ± 150	260 ± 100	660 ± 200

negligible. In ten experiments the mean (± S.D.) hyperpolarization produced by bathing mouse eggs in sodium-free solution was 34 ± 24 mV. A similar hyperpolarization occurred in hamster eggs.

Replacing most of the chloride in the bathing solution by an impermeant anion (see Methods) led to a mean (± S.D.) depolarization of 19 ± 6·6 mV (six mouse eggs); in two hamster eggs chloride replacement led to a depolarization of about 5 mV. Membrane resistance increased in all eggs bathed in low chloride solution.

Previous electrophysiological studies (Powers & Tupper, 1974; Okamoto *et al.* 1977; Miyazaki & Igusa, 1982; Georgiou *et al.* 1983) have reported a slight influence of potassium ions on the resting potential of mouse and hamster eggs. However, these experiments were carried out on eggs depolarized by impalement-leak artifacts and therefore it was necessary to re-examine the effect of potassium on potential and resistance. Raising the potassium concentration from 5 to 40 mM by addition of potassium chloride to a sodium-free solution caused a mean (± S.D.) depolarization of 35 ± 26 mV (six mouse eggs) somewhat smaller than the 52 mV expected from the Nernst equation. In one experiment replacement

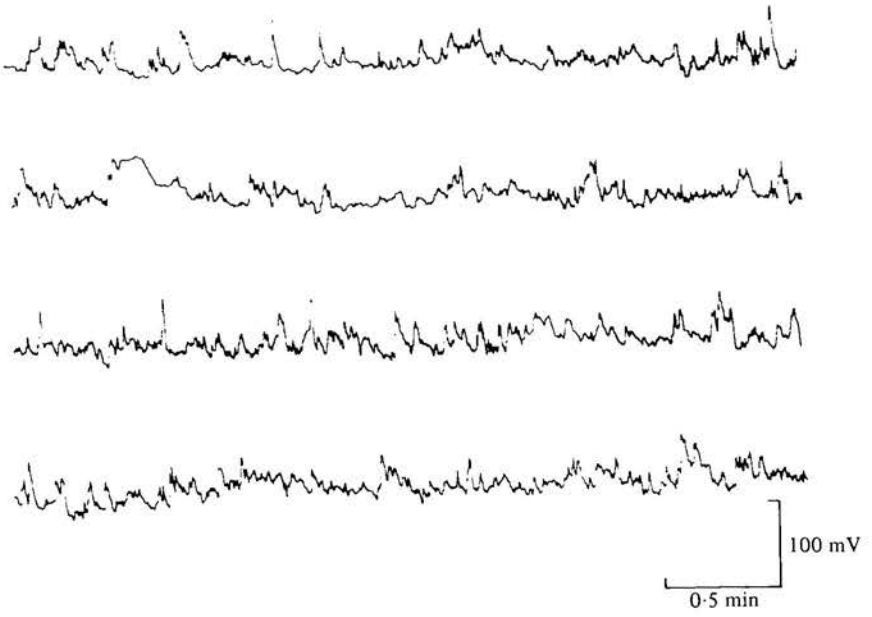

Fig. 2. An intracellular recording from a mouse egg showing spontaneous fluctuations of the membrane potential. The pen traces comprise a continuous excerpt from a longer period of recording. The average membrane potential of this egg was −160 mV and its input resistance was about 1000 MΩ.

of sodium in normal solution by equimolar amounts of potassium to give a final concentration of potassium equal to 25 mM caused a depolarization of 20 mV. Depolarization in all eggs examined was accompanied by a fall in resistance. In all of the above experiments the cells retained their excitability in the solutions with raised potassium concentrations.

An increase of the calcium concentration from its normal value of 4 mM to 40 mM was made in sodium-free solution (Methods). This change usually caused an irreversible depolarization. The input resistance fell during the prolonged depolarizing response to elevated calcium concentration. These results are at variance with previous findings in mammalian eggs (Okamoto *et al.* 1977; Miyazaki & Igusa, 1982). It is possible that the hyperpolarization and increase in resistance observed previously arose from a reduction of the shunt at the tip of the recording micro-electrode.

Active membrane properties

Spontaneous potential fluctuations

Fig. 2 shows a set of continuous intracellular recordings from a mouse egg which exhibits spontaneous fluctuations of membrane potential. Inspection of these traces indicates that the fluctuations are due to waves of depolarization which appear to occur randomly. No detailed analysis of these records has been made. It was observed that the potential fluctuations were accompanied by changes in membrane resistance. In two typical records the resistance fell at the peaks of the depolarizations to about half of its value at the feet of the depolarizing waves. The potential fluctuations were not an invariable feature of eggs with high membrane potentials since they could be selectively abolished by addition of 1 mM lanthanum to the bathing solution. This suggests that they might arise from the random opening of calcium channels since lanthanum ions block voltage-dependent calcium channels (Hagiwara & Byerly, 1981).

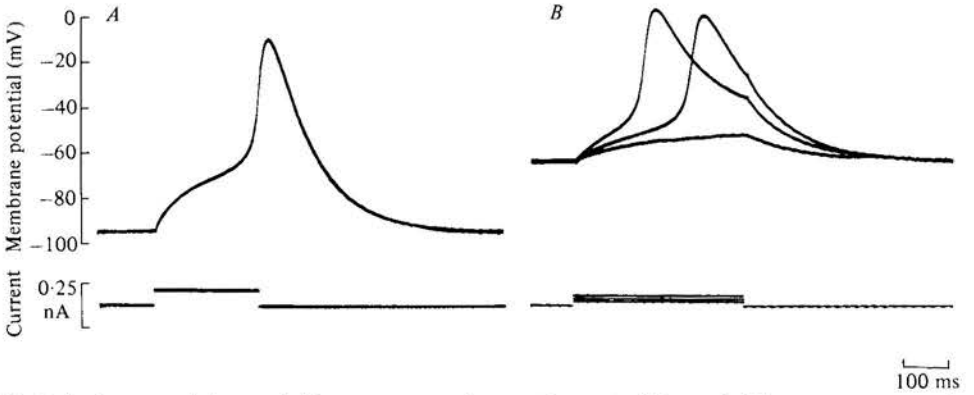

Fig. 3. Action potentials recorded in mouse eggs. *A*, an action potential recorded from a mouse egg stimulated by a depolarizing current pulse (lower trace) passed through the recording micro-electrode. *B*, action potentials in a different egg evoked by depolarizing current pulses of different amplitudes. A subthreshold electrotonic potential is shown also.

Table 3. *Characteristics of action potential in mouse eggs bathed in sodium-free solution*

Egg	Membrane potential (mV)	Threshold (mV)	Action potential peak (mV)	Amplitude of action potential (mV)	Maximum rate of rise ($V.s^{-1}$)
1	−151	−37	+25	176	4·9
2	−131	−57	−6	125	2·1
3	−129	−40	+16	145	2·4
4	−107	−49	+25	132	3·4
5	−132	−57	+4	136	2·2
Mean ± S.D.	−130 ± 16	−48 ± 9	+13 ± 14	143 ± 20	3·0 ± 1·2

Action potentials

A brief depolarizing current pulse passed through the recording micro-electrode evoked an action potential (Fig. 3*A*). In most eggs the action potential was initiated when the membrane potential reached about −50 mV and it attained a positive value at its peak. Measurements were made on action potentials recorded from five mouse eggs to get precise information about threshold, peak value and the maximum rate of rise. In each egg at least four action potentials were analysed and the mean values for the entire study are given in Table 3.

When the amplitude of a depolarizing current pulse sufficient to evoke an action potential was increased the action potential occurred earlier and the peak was increased somewhat (Fig. 3*B*). The increase in the peak value with increase in current strength allowed an estimate of the 'active resistance' to be obtained (cf. Fatt & Ginsborg, 1958). Generally the 'active resistance' at the peak of the action potential was about 100 MΩ as opposed to the 'resting resistance' of about 500 MΩ. This fall in resistance during the action potential indicates the presence of ion channels opened by membrane depolarization.

It was not possible to evoke action potentials at a rate exceeding about 1 Hz. This suggests that the refractory period is about 1 s. Fig. 4 shows a train of action potentials elicited by

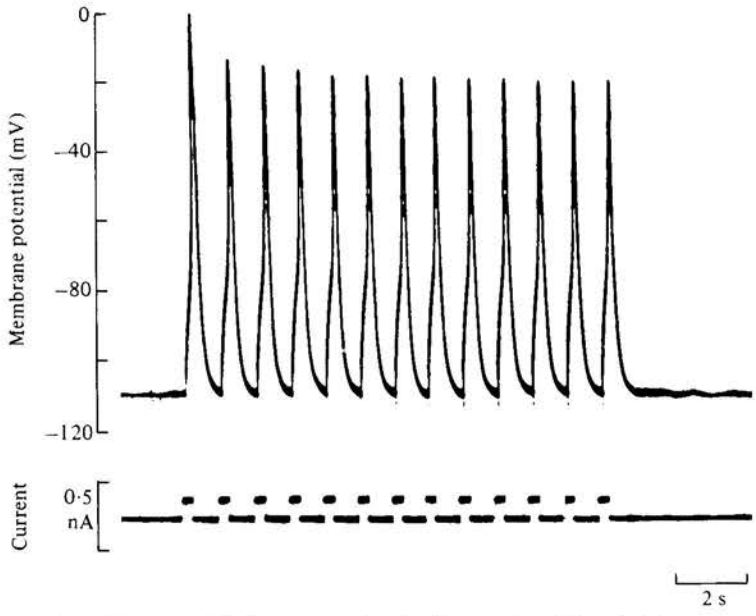

Fig. 4. Successive action potentials (upper trace) evoked by a train of depolarizing current pulses (lower trace) applied to a mouse egg.

a series of depolarizing current pulses. The peak value of successive active potentials declined until a new constant level was reached. Perhaps this decline indicates the presence of a type of channel inactivation similar to that suggested for the inactivation of calcium channels by calcium entry in molluscan neurones (Tillotson, 1979). Another possibility is that during a series of action potentials there is a progressive accumulation of the ion carrying the inward current at the inner surface of the membrane. An explanation of this kind was put forward by Niedergerke & Orkand (1966) to account for the progressive decrease in the overshoot of frog ventricular action potentials, the accumulation of calcium ions causing a fall in the equilibrium potential for calcium.

Ionic basis of action potential

Action potentials could be evoked in mouse and hamster eggs bathed in sodium-free solution (Fig. 5) thus excluding the possibility that the action potential arises from sodium influx. Moreover, the action potential was not reduced in the presence of high concentrations (3 μM) of tetrodotoxin, a sodium channel blocker.

To test whether the action potential is due to the opening of calcium channels two kinds of experiments were performed. In the first the extracellular concentration of calcium was raised from 4 to 40 mM in sodium-free solution. In the second kind the effects of various calcium channel blockers were tested on the egg's action potential.

Fig. 6 shows that raising the calcium concentration in the bathing solution increased both the threshold for initiation of the action potential and the peak value of the action potential in a mouse egg. In similar experiments on ten eggs the mean ($\pm$S.D.) increase in the peak value was $25 \pm 3{\cdot}7$ mV which is not significantly different from the shift (29 mV) expected for a tenfold change in calcium concentration according to the Nernst equation.

It has been established that magnesium ions cannot substitute for calcium as charge carriers in voltage-sensitive calcium channels (Hagiwara & Byerly, 1981). When calcium was

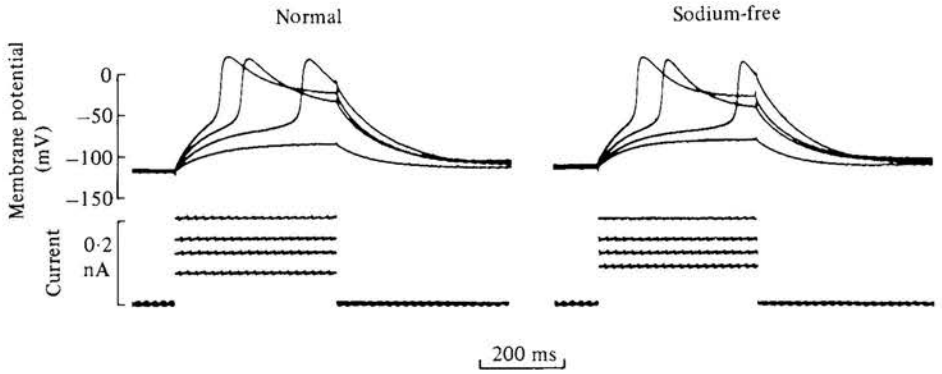

Fig. 5. Action potentials recorded from a mouse egg bathed in normal solution (left) and then in sodium-free solution (right). The records (right) were obtained 7 min after change to sodium-free solution and during the long tail of a spontaneous depolarization; subsequently the resting potential attained −145 mV but peaks of evoked action potentials were similar to those illustrated.

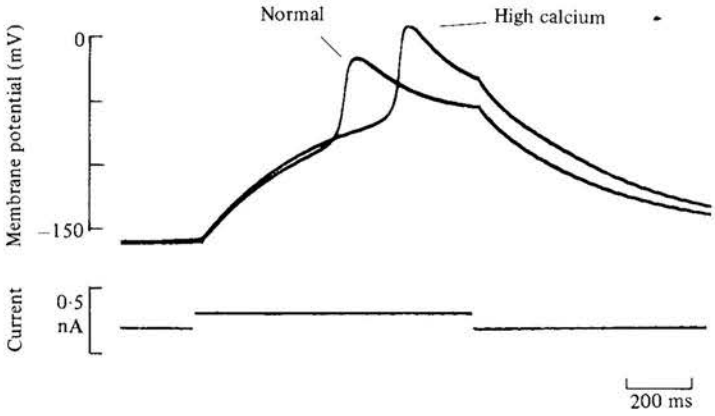

Fig. 6. Effect of raising the extracellular concentration of calcium on the peak value of the action potential recorded from a mouse egg. The calcium concentration was raised from the normal value of 4 mM to 40 mM in a sodium-free solution bathing the egg and the corresponding action potentials have been superimposed.

replaced by magnesium in the solution bathing mouse eggs the size of action potentials was severely reduced. Moreover, the threshold for excitation was raised. Frequently the size of the action potential did not recover fully upon superfusing the eggs with normal solution. However, full-sized action potentials were observed when external calcium concentration was raised to 40 mM.

Calcium channel blockers

Extensive electrophysiological evidence supports the idea that calcium channels can be blocked by a variety of cations including cobalt, manganese and lanthanum and also organic compounds such as verapamil (Hagiwara & Byerly, 1981; Reuter, 1983). It was of interest, therefore, to examine the effects of some calcium channel blockers on the egg's action potential.

Inorganic cations. Cobalt suppressed the action potential when it was present in the

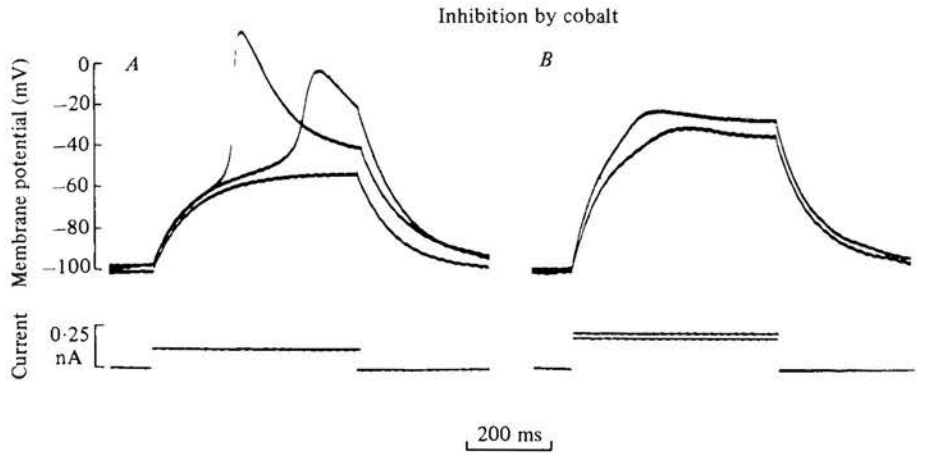

Fig. 7. Effect of cobalt on the action potential recorded from a mouse egg. Superimposed traces of responses to depolarizing current pulses recorded from the egg bathed in normal solution containing 5 mM cobalt chloride. *A*, records show progressive decline of action potentials recorded at 1, 3 and 5 min after solution containing cobalt had entered the chamber. *B*, depolarizations evoked by current pulses of increased amplitude applied to the same egg bathed in normal solution containing 20 mM cobalt chloride. Note that small residual active response persists in the presence of high concentration of cobalt.

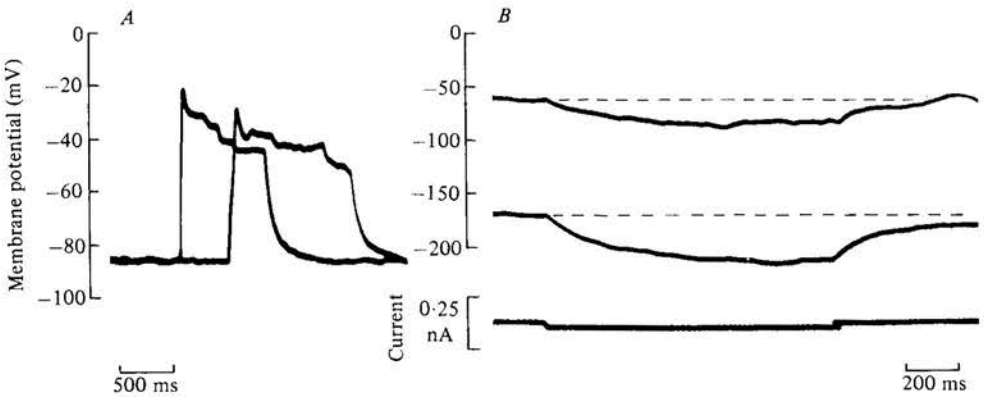

Fig. 8. Spontaneous action potentials recorded from mouse eggs bathed in normal solution. *A*, superimposed spontaneous action potentials in an egg. *B*, electrotonic potentials (upper traces) caused by identical hyperpolarizing current pulses (lower trace) applied to another egg during the plateau of a spontaneous action potential and after its end.

bathing solution at a concentration in the range 5–20 mM (Fig. 7). It is possible that the inhibition of the action potential illustrated in Fig. 7*A* was produced because cobalt raised the threshold for excitation. To examine this possibility the effect of increasing the depolarizing current pulse was recorded. An action potential was not evoked although a small non-linear response remained (Fig. 7*B*). The inhibition caused by cobalt was reversible.

Lanthanum blocked the action potential completely when it was present in the sodium-free solution at 1 mM. This agent also caused a marked hyperpolarization. Recovery was poor unless the bathing solution contained a calcium concentration of 40 mM.

Verapamil. In both mouse and hamster eggs verapamil (10 μM) failed to block the action

potential even after repetitive stimulation. It did produce, however, a 'use-dependence' block at a concentration in the range 200–400 μM as was found at lower concentrations in a voltage-clamp study of the calcium inward current in cat papillary muscle (Ehara & Daufmann, 1978).

Spontaneous action potentials

Occasionally large spontaneous depolarizations were observed. These sometimes attained positive peak values and some had very prolonged plateau phases (Fig. 8*A*). These spontaneous events basically resembled the evoked action potentials and so have been called spontaneous action potentials. Their mean peak value was about -10 mV. It was possible, on a few occasions, to record electrotonic potentials (produced by current pulses through the recording micro-electrode) during and after the plateau phase of a spontaneous action potential. An example is illustrated in Fig. 8*B*. Evidently the membrane resistance is lower during the plateau phase which suggests that a prolonged rise in ionic permeability underlies this phase. The ionic basis of the plateau phase has not been examined because spontaneous action potentials are relatively infrequent and therefore not amenable to controlled studies.

DISCUSSION

The results of this study are at variance with the published reports of other workers in two important respects.

First, we have recorded large membrane potentials and resistances in hamster and mouse eggs. Table 4 shows the available data on the electrical properties on the unfertilized eggs of the mouse, hamster and rabbit. In a previous paper we found that the recorded membrane potential of zona-free hamster eggs lay in the range -9 to -100 mV and that the higher potentials were associated with larger input resistances (Georgiou *et al.* 1983). Evidence was presented to show that low potentials arose from substantial leak artifacts at the point of insertion of the micro-electrode. In the present paper evidence has been presented to demonstrate that impalement produces a drop in potential and resistance (Fig. 1). Provided sufficient time is allowed for sealing of the micro-electrode to the cell membrane the potential and resistance often climb back to their true values. Similar conclusions about impalement-leak artifacts have been drawn from intracellular recordings from eggs of starfish (Miyazaki *et al.* 1975) and sea urchins (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979). Indeed Chambers & de Armendi (1979) found that the application of hyperpolarizing current apparently improved the sealing process at the micro-electrode tip as reported for *Aplysia* neurones (personal communication by E. Mayeri cited by Brown & Flaming, 1977). A possible explanation for the fact that we have obtained apparently better micro-eloctrode sealing than that achieved by other workers studying mammalian eggs is that we usually applied hyperpolarizing current pulses to the cell after impalement.

The second point of difference between our results and those of others on mammalian eggs is that we have recorded action potentials in response to brief depolarizing current pulses whereas they have found evidence for excitability only through anode-break responses. It is likely that the depolarized state of eggs in previous studies has inactivated the voltage-dependent channels. The characteristics of the channels opened by depolarization, namely their ability to pass calcium ions and their block by cobalt, lanthanum or verapamil, indicate that they are selective calcium channels (cf. Hagiwara & Byerly, 1981). The same conclusion has been reached on the basis of the analysis of anode-break responses in mouse and hamster eggs (Okamoto *et al.* 1977; Miyazaki & Igusa, 1982; Eusebi, Colonna &

Table 4. *Electrical measurements on mammalian eggs*

Membrane potential (mV)	Input* resistance (MΩ)	Excitable characteristics	Reference
		Mouse eggs	
−8 to +2	—	—	Cross, Cross & Brinster (1973)
−14	18	—	Powers & Tupper (1974)
−23	50–200	Anode-break response; calcium inward current under voltage clamp	Okamoto *et al.* (1977)
−14	—	—	Fulton & Whittingham (1978)
−7	39	Anode-break response	Yoshida (1983)
−18	11	—	Eusebi & Siracusa (1983)
−22	11	Anode-break response	Eusebi, Colonna & Mangia (1983)
−35	96	—	Jaffe, Sharp & Wolf (1983)
−35	140	—	Igusa, Miyazaki & Yamashita (1983)
−91	430	Action potential	Present study
		Hamster eggs	
−29	150	Anode-break response	Miyazaki & Igusa (1982)
−9 to −100	14–440	Anode-break response	Georgiou *et al.* (1983)
−22	19	—	Eusebi & Siracusa (1983)
−97	410	Action potential	Present study
		Rabbit eggs	
−71	10	—	McCulloh, Rexroad & Levitan (1983)

* Determined from the linear part of the current/voltage relation. Hamster and mouse eggs show rectification above 0 mV and also below −150 mV as found by Miyazaki & Igusa (1982) in cells without action potentials. In the present study the current/voltage relation was linear in the range −70 to −150 mV.

Mangia, 1983; Yoshida, 1983) and voltage-clamp experiments (Okamoto *et al.* 1977; Yamashita, 1982). Calcium channels also occur in the eggs of the starfish and sea urchin (Miyazaki *et al.* 1975; Hagiwara, Ozawa & Sand, 1975; Jaffe, 1976; Chambers & de Armendi, 1979).

Changes in the sodium, potassium, chloride or calcium concentrations in the bathing solution influenced the egg's membrane potential in ways suggesting that the membrane is permeable to these ions. The high value of the egg's membrane resistance, however, indicates that the absolute magnitudes of ionic permeabilities must be low (cf. Powers & Tupper, 1975). It is interesting to note that unfertilized sea urchin eggs also have high resistances (*ca.* 500 MΩ) and that potassium, the most permeant ion in these cells, has a permeability as low as 10^{-8} cm . s^{-1} (Jaffe & Robinson, 1978).

Several features of the action potentials in mouse and hamster eggs deserve comment.

The threshold for excitation is about −50 mV which is about 40 mV more positive than the membrane potential recorded in eggs bathed in normal solution. Fluid in the mouse oviduct, however, contains a higher concentration of potassium, i.e. 25 mM, than that present in our normal solution (Borland, Hazra, Biggers & Lechene, 1977). Our measurements of the effect of extracellular potassium on the membane potential indicate that the membrane potential of eggs in oviducal fluid might be about 20 mV more negative than the threshold potential. Moreover, eggs were excitable in solutions with high potassium concentrations.

The maximum rate of change of membrane potential during the upstroke of the action potential was about 3 V . s^{-1}. This rate is proportional to the positive ionic current entering the egg. The absolute value of this current can be estimated roughly since the membrane

capacitance of the egg has been determined as 1·4 μF . cm^{-2}. So the ionic current entering the egg during the upstroke of the action potential was about 4·2 μA . cm^{-2}. Since the egg's membrane area is apparently about 2×10^{-4} cm^2 the total inward current into the egg was about 0·84 nA. This value is in rough agreement with the size of the inward current produced by depolarizing command pulses in previous voltage-clamp experiments on mouse eggs (Okamoto *et al.* 1977).

In some records (Figs. 3 and 4) the action potential did not overshoot zero. A possibility is that these negative peak values reflect the presence of tip potentials at the recording micro-electrode. However, no such tip potentials were observed on removing the micro-electrode from an egg with an undershooting action potential. Moreover, we recorded large membrane potentials in some eggs and yet these also had overshooting action potentials (Table 3). Although the peak value of the action potential was usually positive it was substantially less than the expected equilibrium potential for calcium (E_{Ca}) as found also in other cells with calcium action potentials (Reuter, 1973). This casts doubt on the strength of the evidence for calcium channels based on a linear relation between the peak of the action potential and the log of extracellular calcium concentration. An alternative view is that the peak of the action potential is more negative than E_{Ca} because an outward positive current occurs as suggested first by Fatt & Ginsborg (1958) for crustacean muscle. It was considered that potassium ions carried this outward current in crustacean muscle. The ionic movements during the action potential in mammalian eggs remain to be investigated in detail. The present electrical evidence suggests the presence of a calcium influx during the upstroke of the action potential.

Does micro-electrode insertion mimic sperm entry?

The progressive rise in potential and resistance which was evident in intracellular recordings from hamster eggs (Georgiou *et al.* 1983) and in the present records from mouse eggs (Fig. 1) might be due to processes initiated by electrode insertion itself rather than to improved micro-electrode sealing as suggested above. Could it be that the insertion of a micro-electrode, in fact, mimics the entry of sperm and that the rise in potential and resistance reflects the process of activation of the egg? This question has been addressed in the literature on eggs of invertebrates which also show a rise in potential and resistance after cellular impalement (e.g. Chambers & de Armendi, 1979). The available evidence based on intracellular recordings from unfertilized and fertilized eggs, on unidirectional flux measurements in unfertilized eggs and on extracellular recordings of 'action currents' at fertilization indicates that micro-electrode insertion does not mimic sperm entry (Jaffe & Robinson, 1978; Chambers & de Armendi, 1979; Whitaker & Steinhardt, 1982, 1983). Unfortunately the corresponding evidence for mammalian eggs is sparse and equivocal.

Powers & Tupper (1975) measured unidirectional fluxes of sodium, potassium and chloride ions in unfertilized mouse eggs. Assuming low values of the membrane potential they estimated ionic permeabilities and hence ionic conductances. The estimated total ionic conductance was 880 μS . cm^{-2} whereas the measured membrane conductance was 380 μS . cm^{-2} (Powers & Tupper, 1974). The data in this paper give a membrane conductance of about 10 μS . cm^{-2}. Thus there exists a serious disparity between the ion flux determinations and our electrical measurements. The disparity needs experimental investigation and at present it excludes any useful argument about the size of the potential and resistance of eggs on the basis of their ionic fluxes.

It is crucial to establish the potential and resistance of fertilized eggs. It is our contention, however, that all previous recordings from fertilized mammalian eggs probably suffered from impalement-leak artifacts. In hamster eggs fertilization is accompanied by a series

of transient hyperpolarizations and transient rises in conductance (Miyazaki & Igusa, 1981, 1982; Igusa & Miyazaki, 1983). In mouse eggs fertilization is accompanied by a small hyperpolarization (Igusa, Miyazaki & Yamashita, 1983; Jaffe, Sharp & Wolf, 1983) and in rabbit eggs by a slow depolarization and repetitive diphasic potential changes (McCulloch, Rexroad & Levitan, 1983). Since no investigator has reported a rise in resistance accompanying fertilization it seems highly unlikely that the changes we have reported in the electrical properties of hamster and mouse eggs upon impalement reflect an activation process started by micro-electrode penetration. Moreover, when a second micro-electrode is inserted into a hamster or mouse egg after the high potential and resistance state is attained there is always a transient fall both in the potential and the resistance of the egg (Fig. 4, Georgiou *et al.* 1983; Fig. 1, present study) as would be expected for a transient leak pathway at the micro-electrode tip.

Finally, strong indirect evidence that insertion of a recording micro-electrode cannot activate mammalian eggs comes from a detailed study by Uehara & Yanagimachi (1977). These authors activated hamster eggs by piercing them with fine glass needles. Small needles with diameters less than 2 μm were ineffective at causing activation whereas needles with diameters in the range 3–5 μm were effective provided several piercing movements were made. Since the piercing movement amounted to pushing the needle through the egg till it emerged at the other side it is highly unlikely that the relatively gentle insertion of a fine-tipped micro-electrode can activate mammalian eggs. Moreover, Fulton & Whittingham (1978) have reported that insertion of micropipettes with fine tips ($\ll 0{\cdot}5$ μm) did not activate mouse eggs.

Function of calcium action potential

It is possible that sperm entry into a mammalian egg evokes an action potential since a similar finding has been made in the eggs of sea urchins (Jaffe, 1976; Chambers & de Armendi, 1979). In sea urchins, however, it is by itself not a sufficient stimulus for egg activation and indeed activation can occur in the absence of action potentials (see Whitaker & Steinhardt, 1982). An action potential occurring during the fertilization of a mammalian egg might serve as an appropriate trigger for exocytosis of the material in the cortical granules by virtue of the calcium influx which must occur. It is doubtful whether sufficient calcium will flow in during an action potential to produce a widespread rise in the cytosolic calcium concentration. However, it is possible that the rise in cytosolic calcium concentration arising from an action potential could cause secondary calcium release from intracellular stores (Gilkey, Jaffe, Ridgway & Reynolds, 1978). Evidence from studies with aequorin suggests that fertilization of mouse eggs is accompanied by periodic rises in the cytosolic calcium concentration (Cuthbertson, Whittingham & Cobbold, 1981). Moreover, Fulton & Whittingham (1978) have shown that calcium ionophoretically injected into mouse eggs causes parthenogenetic activation. The possible roles that the action potential plays in the processes of the cortical reaction and the polyspermy block remain to be investigated and the present paper describes results suggesting that such investigations could be worthwhile.

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Calcium injection into hamster eggs causes hyperpolarization and decrease in membrane resistance

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When a sperm attaches to a hamster egg the egg's plasma membrane undergoes a transient hyperpolarization and a fall in resistance (Miyazaki & Igusa, 1982). Moreover, ionophoretic injection of calcium ions into hamster eggs evokes a similar hyperpolarization although there has been no direct test that calcium also produces a reduction in membrane resistance. We report here that calcium injection into hamster eggs causes a large decrease in membrane resistance.

Eggs were obtained by superovulation from mature female golden hamsters. After removal from the oviducts the eggs were bathed at room temperature (20–22 °C) in a solution containing (mM): NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1·2; glucose, 5·6; Na lactate, 20; Na pyruvate, 1; Tris-Tris Cl pH 7·6, buffer 5. Bovine serum albumin (4 mg/ml) was added to the solution. Cumulus was removed by bathing eggs in solution containing Hyaluronidase (1 mg/ml) for 2–4 min and then the zona pellucida was removed by transfer into a solution containing Trypsin (1 mg/ml) for 1–3 min.

A micro-electrode (50 MΩ) filled with 2 M-K acetate was inserted into an egg for potential recording; current pulses were passed through this intracellular electrode by a bridge circuit for input resistance measurement. Calcium was injected ionophoretically into the egg by passing current pulses (0·2–5 nC) through a second intracellular micro-electrode (10 MΩ) filled with 1 M-$CaCl_2$.

Intracellular recordings have been made in twenty hamster eggs. The mean ± S.D. values for resting potential and input resistance were -32 ± 14 mV and 135 ± 55 MΩ respectively. Upon calcium injection each egg underwent a transient hyperpolarization lasting about 30 s and a transient fall in its input resistance which usually outlasted the potential change. The mean values (± S.D.) for the minimum membrane potential and input resistance during the response to calcium were -53 (± 13) mV and 44 (± 29) MΩ. Thus intracellular calcium injection produces a large fall in membrane resistance. Analysis of the electrical response according to a simple equivalent circuit model (Trautwein & Dudel, 1958) yields a reversal potential of -73 ± 13 mV (mean ± S.D.).

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Action potential in unfertilized mouse egg

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Previous work on unfertilized mouse eggs (Powers & Tupper, 1974; Okamoto, Takahashi & Yamashita, 1977) has yielded low resting potentials (−14 to −45 mV), input resistances in the range 20–200 MΩ and anode-break responses to hyperpolarizing current pulses. However, these intracellular recordings probably suffered from impalement leak artefacts, because we have found that after allowing a suitable period for sealing of the micro-electrode to the cell membrane, large membrane potentials of −80 mV and input resistances of about 500 MΩ are observed. In this condition action potentials occurred in response to brief depolarizations.

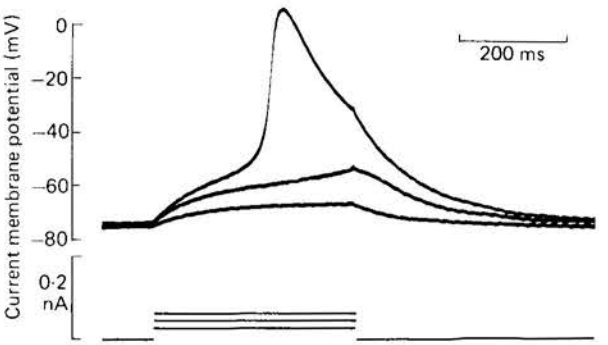

Fig. 1. Superimposed intracellular recordings from an egg. Upper traces are responses to current pulses (lower traces) passed through the recording micro-electrode (50 MΩ) filled with 2 M-K acetate. The egg was bathed at 24 °C in a solution (pH 7·3) containing (mM): choline Cl, 120; KCl, 5; $CaCl_2$, 4; $MgCl_2$, 1·2; glucose, 5·6; HEPES, 5; NaOH, 2·5. Methods of obtaining zona-free eggs from mice have been described by House & Bland (1983).

Action potentials have been recorded from zona-free eggs (Fig. 1) and also from eggs with zona pellucida. The peak value of the action potential depended on the external concentration of calcium but was independent of sodium concentration. Cobalt chloride (5 mM) in the bathing solution caused an almost complete block of the action potential and this block was reversible. We conclude that the mouse egg has a calcium-dependent action potential.

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Single-channel currents in unfertilized mouse eggs

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The presence of calcium channels in unfertilized mouse eggs is indicated by calcium action potentials evoked by depolarization (Georgiou, Bountra, Bland & House, 1984) and by inward calcium currents in voltage clamp experiments (Okamoto, Takahashi & Yamashita, 1977).

In order to examine these channels further, cell-attached patch recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) have been made from zona free mouse eggs. To facilitate giga-seal formation and to enhance the mean amplitude of single-channel currents, the solution bathing the egg and filling the patch electrode contained 80 mM-$SrCl_2$ as a replacement for NaCl. When the patch electrode was held near to 0 mV (transpatch potential is then about −90 mV), spontaneous inward currents were observed (Fig. 1).

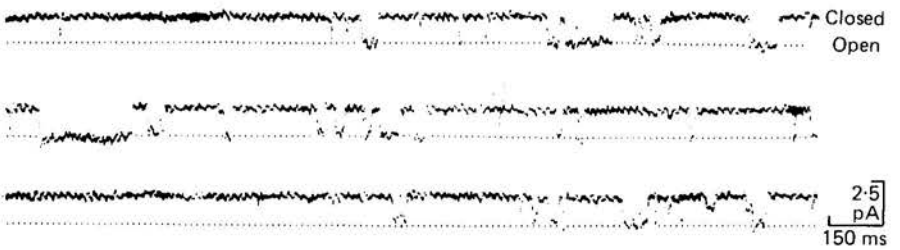

Fig. 1. Inward currents at a patch potential 12 mV more negative than the cell's resting membrane potential (filtered at −3 dB 100 Hz). The egg was bathed at 24 °C in a solution (pH 7·2) containing (mM): $SrCl_2$, 80; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1·2; glucose, 5·6; lactate, 20; buffered using Hepes, 5, and NaOH, 2·5. Methods of obtaining zona free eggs have been described by Georgiou *et al.* (1984).

At rest the channels were seen to open in bursts, as has been found in other preparations containing calcium channels (Reuter, 1983). The mean ± S.D. values of the amplitude of these channel currents were found to be 1·3 ± 0·5 pA ($n = 342$); probability of opening was 0·18. Furthermore channels open in synchrony with evoked action potentials.

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